AD_____

Award Number: DAMD17-98-1-8282

TITLE: Activation of Estrogen Receptor-Beta-Dependent Transcription by Estrogen-Independent Pathways

PRINCIPAL INVESTIGATOR: Carolyn L. Smith, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: September 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

20020329 201

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2001	3. REPORT TYPE AND DATES COVERED Final (1 Sep 98 – 31 Aug 01)

4. TITLE AND SUBTITLE
Activation of Estrogen Receptor-Beta-Dependent Transcription by Estrogen-Independent Pathways

5. FUNDING NUMBERS
DAMD17-98-1-8282

6. AUTHOR(S)
Carolyn L. Smith, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-Mail:

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

There are two known receptors for estrogens, ERα and ERβ. The existence of ERβ was only recently appreciated, and little is understood about its ability to be activated by intracellular signaling pathways in the absence of estrogens. The purpose of this research program is to characterize the ability of ERβ to by activated by various ligand-independent signaling pathways, and to characterize the structural regions of ERβ, in comparison to ERα, that regulate how this receptor subtype responds to intracellular cross-talk. We have found that stimulation of HeLa cells with forskolin and IBMX results in the activation of ERα and ERβ dependent expression in a receptor-dependent and promoter context-dependent manner, and that protein kinase A mediates this response. Factors that interact with an AP-1 binding site contribute to forskolin/IBMX activation of estrogen receptor-dependent gene expression, and do so in a manner that does not require the A/B domain of either receptor. At least c-Jun is able to stimulate ERα activity via the AP-1 binding site. Multiple coactivator proteins, predominantly of the steroid receptor coactivator (SRC) family and CREB binding protein (CBP) can stimulate ERα and ERβ activity induced by forskolin/IBMX pathways indicating that these coactivators can functionally interact with these receptors in the absence of ligand. Coactivation, however, does not appear to require SRC1 phosphorylation as has been shown to be the case for progesterone receptors. This suggests that multiple pathways can be employed to regulated steroid receptor transcriptional pathways in a ligand-independent manner, and illustrates that understanding the mechanisms that regulate ERα and ERβ transcription activity provides insight into how transcription factor cross-talk can be regulated by a single agent.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
98

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

Introduction

Breast cancer remains the major cancer (excluding skin cancer) among women in the United States with more than 180,000 new cases anticipated in 2000. The requirement of estrogens for normal breast development is well documented (1). However, estrogens also have been linked to breast cancer, presumably through their ability to stimulate cell proliferation (2) and inhibition of estrogen action therefore has been a primary objective in the treatment, and more recently the prevention of, breast cancer. For many years, estrogen effects were thought to be mediated by a unique, high affinity intracellular receptor protein, the estrogen receptor (ER), that is a member of a superfamily of transcription factors (3, 4). The basic mechanisms of ER activity have been ascertained. Hormone binding to ER results in receptor homodimerization and binding to specific enhancer DNA elements located in the promoter regions of target genes (5, 6). This process, which is accompanied by increases in ER phosphorylation (7-12), enables "activated" receptors to regulate the transcription of hormone-responsive target genes and the resulting changes in mRNA and protein synthesis are ultimately responsible for alterations in cellular function. The structural features of the estrogen receptor (ERα) responsible for hormone binding, dimerization, DNA binding and transcriptional activation have been identified (3, 13-16) and these studies have provided the basis of our understanding of the molecular mechanisms by which estrogens regulate the growth and differentiation of mammary tissues.

Clearly, the transcriptional activity of the ER can be regulated by estrogens, such as 17β-estradiol (E2). However, the ERα also can be activated in the absence of exogenous ligand by agents that stimulate intracellular signal transduction cascades (EGF, IGF-1, heregulin, dopamine, TPA and cAMP) (7, 17-23) or inhibit protein phosphatases (okadaic acid) (19). Furthermore, cyclin D1, independent of cyclin-dependent kinases, also can activate the ER in the absence of estrogen (24). The ERα knock-out mouse model confirms that ERα is required for some but not all *in vivo* EGF effects and established the importance of ligand-independent activation of ER to physiological events (25). Most of these ligand-independent activation pathways (with the exception of cyclin D1) increase receptor phosphorylation (7, 12, 23, 26, 27) and mutation of the only known ligand-independent (EGF) phosphorylation site (serine[118]) to an alanine residue abolishes EGF activation of the ER (28), suggesting that phosphorylation may play an important role in these activation pathways. However, this point mutant does not block cAMP-mediated gene expression and different domains are required to respond to EGF and cAMP signaling pathways (29), suggesting that multiple mechanisms must exist to enable ER to activate target gene expression in response to diverse regulatory events.

In 1996, a new member of the nuclear receptor superfamily was cloned from a prostate cDNA library (30). When the resulting cDNA was sequenced and expressed, it became apparent that a novel estrogen receptor had been identified. This new member of the nuclear receptor superfamily was named ERβ, and the original estrogen receptor was renamed ERα. The ERβ binds to estradiol with an affinity (K_d 0.4 nM) similar to ERα and binds to the same DNA response element as ERα (30-32). Thus, it is reasonable to predict that ERβ regulates the expression of at least a subset of ERα target genes. However, the relatively undeveloped mammary glands in the ERα knock-out mouse indicate that ERβ is not equivalent to ERα (33). The reasons for this are unclear, but could be related to differential expression and/or differences in the ability of α and β estrogen receptors to activate target gene expression. Mouse, rat and human ERβs are approximately 65 amino acids smaller than their corresponding α-receptors,

and the A/B, D and F domains (**Figure 1**) are poorly conserved (30, 32, 34). Furthermore, the ligand binding domains (domain E) of ERα and ERβ are only ~55% identical and rat ERα and ERβ receptors do not bind equally well to all ligands (31). The expression patterns of ERα and ERβ mRNAs are different but overlapping (31) suggesting that the genes for ERα and ERβ are independently regulated. However, both ERα and ERβ mRNA have been detected in human mammary gland, breast tumors and several human breast cancer cell lines (35, 36). Taken together, these data suggest that ERβ is likely to play a role in mediating estrogen action in mammary gland, but that this receptor is unlikely to be functionally equivalent to ERα.

The identification of a second estrogen receptor raised a number of important biological questions such as, what is the expression of ERβ, relative to ERα, in normal and malignant mammary tissue? However, knowledge regarding the expression of ERβ in mammary gland will be of limited value without detailed information on the transcriptional activity of ERβ. Do ERα and ERβ respond similarly to ligand-independent pathways? Are currently used antiestrogens equally effective antagonists of ERα and ERβ? Do ERα and ERβ activate the same target genes to a comparable extent? Studies of this nature will provide the information necessary to determine whether resources are required to develop new strategies to more effectively and/or selectively block ERα- and ERβ-mediated estrogen effects. Indeed, increasing evidence demonstrates that ERα and ERβ are not functionally equivalent, and our experiments reported below contribute to the foundation upon which new strategies to regulate ERα and ERβ biological activity can be developed. Moreover, comparing and contrasting the structure/function relationships of ERα and ERβ with respect to activation by ligand-independent pathways represents a novel approach to study mechanistic questions relating to activation of gene expression in the absence of estrogens.

Body

A new member of the nuclear receptor superfamily, ERβ, has been identified that binds to estrogens with high affinity, and binds to the same DNA response elements as the classical estrogen receptor, ERα. Both of these ligand-regulatable transcription factors possess a well-defined, centrally located, DNA binding domain and carboxy-terminal domain, which contains a ligand-dependent activation function (AF-2); however the amino terminus which possesses a second activation function (AF-1) is poorly conserved. Thus, it is highly likely that that the biological activity of ERβ will differ from that of ERα. This hypothesis is being tested in the following two technical objectives:

1. To determine if estrogen-independent signaling pathways can stimulate ERβ transcriptional activity.

2. To determine what regions of ERβ contribute to its estrogen-independent transcriptional activity and to compare these regions to known ERα activation functions to characterize the structural features of these receptors that contribute to their respective biological properties.

The originally reported form of ERβ represented a truncated version of the subsequently identified full length form of the receptor. In the first year of this award, we conducted experiments directed towards resolving differences in activity and expression of the full-length

and truncated forms of ERβ. All of our studies in years 2 and 3 utilized the full-length form of ERβ, unless deletion mutations were being analyzed.

In the second year, we continued our analyses of the ability of ERβ to be activated by ligand-independent, cAMP-stimulated, signaling pathways. Both ERα and ERβ are activated in cells treated with forskolin and isobutylmethylxanthine (IBMX). Forskolin is an activator of adenylyl cyclase and IBMX is a phosphodiesterase inhibitor, and treatment of cells with these compounds therefore results in an increase in intracellular cAMP levels. In transient transfection assays, 10 μM forskolin and 100 μM IBMX stimulated the ERβ activation of ERE-Elb-CAT target gene by ~6-fold while ERα-dependent gene expression was stimulated by ~3-fold. Minimal change in target gene expression was observed in cells transfected with the reporter gene and an empty expression vector indicating that the increased CAT activity is receptor-dependent. Furthermore, an ERα mutant possessing point mutations in its DNA binding domain (C201H/C205H) was unable to mediate forskolin/IBMX-induced CAT gene expression indicating that receptor binding to DNA was required. Further analysis demonstrated that a target gene lacking the ERE could not be stimulated by the forskolin/IBMX-induced signaling pathway in cells expressing ERα or ERβ. Thus, intracellular cAMP signaling pathways have the potential to activate the transcriptional activity of both ERα and ERβ, and this activation is dependent on the expression of an estrogen receptor, the receptor's ability to bind to DNA and the presence of an estrogen response element within the target gene. To ensure that target gene expression resulted from forskolin/IBMX activation of the cAMP-dependent/protein kinase A (PKA) signaling pathway and not a non-specific event, we demonstrated that the specific protein kinase inhibitor, H89, blocked forskolin activation of both receptor isotypes, but not transcriptional activity stimulated by E2, supporting the hypothesis that activation occurs via a cAMP/PKA dependent signaling pathway.

The preceding experiments were performed with the ERE-Elb-CAT or ERE-tk-CAT target genes which consist of an estrogen response element linked to a TATA box or thymidine kinase promotor, and the CAT reporter gene. To investigate whether the target gene influenced the ability of forskolin/IBMX to activate receptor-dependent gene expression, the same experiment was repeated, but using other target genes. As we also demonstrated previously, the expression of the pS2-CAT, pATC0, pATC1, pATC2, ERE-Elb-Luc and pC3-Luc target genes was not stimulated by forskolin/IBMX, although E2 increased gene expression in every target gene that possessed an ERE. In contrast, the activity of the ERE-tk-CAT, ERE-Elb-CAT and pC3110-tk-Luc target genes was increased by forskolin/IBMX. In year 3, we also examined forskolin/IBMX induction of the oxytocin promoter and found that the cAMP pathway stimulated ERα but not ERβ transcriptional activity (**Figure 2**). This indicates that the ability of the cAMP-dependent PKA pathway to activate target gene expression was dependent on the nature of the reporter gene examined. The majority of these target genes contain consensus EREs, so we therefore turned our attention to other potential transcription factor binding sites that are present within the synthetic target gene vectors, in order to determine what role, if any, they play in forskolin/IBMX activation of ER-dependent gene expression.

Many vectors have an imperfect AP-1 binding site (also known as a TPA responsive element or TRE) located several hundred base pairs upstream of their minimal promoters. Both the ERE-Elb-CAT and ERE-tk-CAT vectors have such a site. In order to determine if this binding site contributed to the overall activation of gene expression following forskolin/IBMX stimulation of cells, we made a four nucleotide insertion within the putative TRE of ERE-Elb-CAT that

prevents AP-1 from binding to DNA (37). Interestingly, forskolin/IBMX was unable to activate ERβ-dependent expression of the resulting mutated target gene even though E2 could still stimulate ERβ activity. In contrast, mutation of the putative AP-1 site did not block forskolin/IBMX activation of target gene activity by ERα, although it decreased the relative magnitude of the response. Similar results were obtained when the AP-1 site was removed through a more extensive deletion of 195 bp surrounding the AP-1 binding site. These experiments suggested that AP-1 sites were contributing to the ability of ERα and ERβ to stimulate ERE-dependent gene expression, and that factors that bound to the TRE and ERE sites were cooperating to bring about activation of transcription. In support of this, we and others (38, 39) have shown that forskolin/IBMX activates AP-1-dependent gene expression. Although the reporter genes that we have used in our studies are by their very nature, synthetic in origin, it is interesting to note that TREs are widely distributed in the promoter region of many endogenous genes, including the progesterone receptor which has been shown to be stimulated in an ER-dependent manner by treatment of cells with IBMX and cholera toxin (40), an agent that like forskolin/IBMX will stimulate intracellular cAMP production/accumulation and activation of a PKA signaling pathway, or forskolin (41).

It has been reported previously that ERα can interact with c-Jun, one component of the AP-1 transcription factor directly through the receptor's A/B domain (42). However, both ERα and c-Jun are also able to bind to coactivators, such as CBP/p300 (43-45), and it is possible that the cooperative functional interactions between these two transcription factors are direct (*e.g.* they bind to one another) or indirect (*e.g.* they interact via association with a common coactivator). To begin to distinguish between these two possibilities, we analyzed the ability of ERα and ERβ deletion mutants lacking their A/B domains to be activated by the forskolin/IBMX-stimulated signal transduction pathway. In our first experiment, the A/B domains of ERα and ERβ were deleted to generate expression vectors for ERα-179C and ERβ-143C, respectively. These deletion mutants were tested for their ability to activate the expression of the ERE-Elb-CAT target gene in response to the forskolin/IBMX-induced signaling pathway. Forskolin/IBMX activated the transcriptional activity of ERα-179C and ERβ-143C, the former to an extent reduced in comparison to its respective wild type receptor. However, when assays were performed with the mutated target gene, ERE-Elb-CAT (mTRE) in which the TRE has been disrupted by a four nucleotide insertion within the putative AP-1 site, neither receptor deletion mutant was able to stimulate transcription of the target gene. Taken together, this indicates that activation of target gene transcription by the cAMP-dependent/PKA signal transduction pathway requires an estrogen receptor as well as another activity dependent on the TRE site, and that these interactions do not require the A/B domain of either ERα or ERβ.

Our experiments in the third year have continued to examine the ability of cAMP signaling pathways to stimulate the activity of ERα and ERβ. Because our studies in year 2 demonstrated that the amino-terminus of ERα contributed to this response (see above), and because phosphorylation of this region of ERα contributes to AF-1 activity, we examined whether mutation of the amino-terminal phosphorylation sites affected the ability of forskolin/IBMX to stimulate gene expression. As shown in **Figure 3**, when three of the major ERα amino-terminal phosphorylation sites are mutated to alanine residues, the ability of forskolin/IBMX to stimulate transcription, relative to E2-induced gene expression is maintained. It was apparent, however, that these mutations decreased the overall ability of estradiol or forskolin/IBMX to stimulate activity; the former result was expected from previous findings (10, 46). This indicated that

these phosphorylation sites were not specifically required for activation of ERα transcriptional activity by forskolin/IBMX.

We have concentrated a significant amount of effort examining the ability of proteins to facilitate the interaction between ERs and the factors that bind to the TRE site. Our assumption in our year 2 studies had been that the factor that binds to the TRE site was AP-1. AP-1 is composed of either homo- or heterodimers within the Jun family (c-Jun, JunB and JunD) or between members of the Jun and Fos (c-Fos, FosB, Fra1 and Fra2) families (47). We therefore examined whether AP-1 proteins could functionally interact through the TRE site. HeLa cells were transfected with an ERα expression vector and a reporter gene with (TRE-ERE) or without (ΔNde-Eco-ERE) the TRE site. In addition, these cells were transfected with expression vectors for either c-Jun, c-Fos or combinations of the two. Our data demonstrate that c-Jun is able to significantly increase CAT gene expression only when a TRE site was present in the vector (Figure 4A). c-Fos on its own had little effect on the magnitude of gene expression, and had little effect on c-Jun activity (Figures 4B & C). Thus, the AP-1 protein c-Jun can stimulate ERα activity only when a binding site (TRE) for this protein is present. Moreover, this result demonstrates that AP-1 proteins are able to exert the TRE-dependent effects on ERα activity that we have observed.

Since c-Jun is known to bind to ERα via its amino-terminus, and since this domain is dispensable for cAMP activation as long as there is a TRE site in the vector, we hypothesized that another molecule must act as a bridging factor between these two transcription factors. This putative factor would need to be able to interact with both c-Jun as well as ERs. Furthermore, this putative factor would need to functionally interact with both ERα and ERβ through the carboxy-terminal portion of either receptor. We have now evaluated a number of candidates. The first is a coactivator of c-Jun and steroid receptors called JAB1 (48). When JAB1 was cotransfected into cells, it very modestly enhanced the activity of both ERα and ERβ stimulated by either forskolin/IBMX or E2 regardless of whether a TRE site was present in the promoter (Figure 5A & B). Due to the relative lack of JAB1 activity in this system and the independence of its effects from the TRE site, we have not pursued this further. We also considered the activity of cyclin D1, a protein which has been implicated in the activation of ER by virtue of its ability to promote SRC-1 and P/CAF recruitment to the receptor (49, 50). In addition, it had been recently shown that cAMP treatment of cells enhanced the interaction between ERα and cyclin D1 (51). Surprisingly, we found that coexpression of cyclin D1 in our cells resulted in a decrease in ERα-dependent gene expression, contrasting with the results of others (Figure 6). We have repeated this experiment multiple times and the result is consistent. It is possible that differences in cellular environment may contribute to these differences. However, since cyclin D1 overexpression was not able to enhance ERα and ERβ transcriptional activity stimulated by forskolin, this factor seems unlikely to be a contributing factor facilitating cAMP interactions between AP-1 and ERs.

One final approach that we have employed is to develop a dominant negative form of CBP. CBP is a coactivator for both c-Jun (45) and ERα (52). We have made use of the fact that the region of CBP required for interaction with c-Jun (amino acids 461 to 661) is distinct from the residues required to interact with ERα (amino acids 1-101) (53). Thus, by creating an expression vector for the c-Jun interaction region (JIR) one would expect to block the ability of CBP to interact with c-Jun, but not with ER. When the JIR fragment was overexpressed in cells, we

observed a decrease in ERα and ERβ activity both stimulated by E2 as well as by forskolin/IBMX pathways (**Figure 7**). On an ERE-E1b-CAT reporter, this would be expected since as we showed in figure 4, that the TRE site contributes to E2 and forskolin/IBMX activity. We are currently confirming the expression of the JIR fragment, and will then proceed to examine its effects on a reporter lacking the TRE site.

We had previously shown that SRC-1 overexpression could coactivate ERα activity stimulated by forskolin/IBMX (54). To confirm and extend this observation, we examined the ability of SRC family and the CBP coactivators to stimulate that transcriptional activity of ERα and ERβ. As shown in **Figure 8**, all four coactivators could stimulate the activity of both receptors whether activated by either E2 or forskolin/IBMX. Moreover, this coactivation could be observed in the absence of the TRE site, indicating that interaction with ER alone is sufficient to allow the coactivators to stimulate ligand-independent (cAMP) ER-dependent gene expression (data not shown). It has been recently published that cAMP treatment of cells results in phosphorylation of the SRC-1 coactivator, and that this phosphorylation is associated with a increase in the ability of coactivator to stimulate the activity of the chicken progesterone receptor stimulated with either progesterone or 8Br-cAMP, another agent capable of initiating cAMP signaling pathways (*e.g.* activation of PKA) in cells (55). We therefore examined the ability of SRC-1 phosphorylation mutants to stimulate gene expression induced by treatment of cells with forskolin/IBMX. As shown in **Figure 9**, mutation of the SRC-1 phosphorylation sites does not block cAMP activation of ER-dependent gene expression. This is important because it indicates that cAMP activation of the transcriptional activity of different members of the steroid receptor superfamily (*e.g.* ER and PR in this case) may be achieved by multiple mechanisms, and that various components of the steroid receptor gene expression pathway can be targeted for cAMP regulation. This also sheds light on how it is possible that activation of ERα and ERβ may be distinct.

We also initiated studies in year 3 to examine the ability of the dopamine pathway, which has been shown to ligand-independently activate ERα (17), to activate ERβ transcriptional activity. For these studies we used a synthetic full dopamine receptor agonist, SKF-82958 (±-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrehydro-1H-3-benzazepine; see **Figure 10A**), since this compound is more stable than dopamine. As previously reported (56), SKF-82958, like dopamine, stimulated ERα transcriptional activity, and this was inhibited by the pure ER antagonist ICI 182,780 (**Figure 10B**). We then examined activation of human ERβ using the ERE-E1b-Luc reporter gene. SKF-82958 was not able to significantly activate ERβ-dependent gene expression in comparison to the ability of this compound to stimulate ERα transcriptional activity as shown in **Figure 11A** or in dose response studies (data not shown). SKF therefore appears to preferentially activate ERα. To ensure that SKF-82958 induction of ERα-dependent gene expression was not due to ligand stabilization of ERα expression, Western blot analysis of ERα expression in cells treated with vehicle, E2 and SKF-82958 was performed, and like E2 and dopamine (17, 57), SKF was found to down-regulate the expression of ERα in HeLa cells (**Figure 11B**). Dose response studies indicated that half-maximal induction of ERα-directed gene expression by SKF-82958 occurred at 2 μM (data not shown). In contrast, maximal dopamine induction of ER-directed gene expression occurs at 100-250 μM (17, 19, 28), suggesting that SKF-82958 is a more potent activator of this response. However, the potency (K_m) and maximum efficacy of SKF-82958 induction of cAMP are similar to that for dopamine

in rat brain striatum after treatment in vivo (58). This discrepancy suggested that there may be mechanistic differences in the ability of SKF-82958 and dopamine to stimulate ERα transcriptional activity.

To investigate this further, SKF stimulation of cAMP production in HeLa cells was examined by RIA and compared to the ability of SKF to activate ER-dependent gene expression. No correlation was found, as micromolar doses of SKF-82958 failed to significantly elevate cAMP levels (data not shown). To more closely mimic conditions under which our transactivation assays are performed, the ability of SKF-82958 to stimulate cAMP response element (CRE)-dependent transcription was assessed. The -169αCG-CAT gene is composed of a fragment of the human chorionic gonadotropin gene promoter containing a CRE element, linked upstream of the CAT reporter gene and is activated by cAMP stimulation of the CREB transcription factor (59). The -100αCG-CAT reporter gene which lacks the CRE was used as a negative control. As shown in **Figure 12**, CRE-dependent transcription was stimulated by 8Br-cAMP and, more modestly, by dopamine. However, there was no stimulation of CRE-dependent transcription by E2 or SKF-82958. These results suggest that SKF-82958 is not acting through stimulation of a cAMP-dependent dopaminergic signaling in this system. This result led to a consideration of whether this compound activated ER-dependent gene expression through direct binding to ERα. This question is further underscored by the ring structure of this synthetic D1 receptor agonist which is reminiscent of the structures of some ER agonists and antagonists (60).

In order to determine whether SKF-82958 could bind to ERs, whole cell competitive hormone binding assays were performed in HeLa cells transfected with expression vectors for either ERα or ERβ. Cells were incubated with [^{3}H]estradiol and increasing amounts of unlabeled E2, SKF-82958 or dopamine. The displacement curves for ERα and ERβ indicate that SKF-82958 can compete weakly with estradiol for binding to both forms of ER but that dopamine is unable to do so (**Figures 13A & B**). The average relative binding affinities of SKF-82958 in comparison to E2 (100) for ERα (0.077 ± 0.018; n=4) and ERβ (0.069 ± 0.009; n=3) are similar and are comparable to those measured by other investigators for low affinity ER agonists such as bisphenol A (31). This result suggests that activation of ERα-dependent gene expression may arise through SKF-82958 binding to ERs and serving as a weak receptor agonist. Therefore, instead of SKF ligand-independent activation of ERα and not ERβ, it appears that SKF bind to both ERs and acts as a ERα subtype selective agonist. The identification of these types of compounds are important because this contributes to the ongoing identification and utilization of subtype selective ligands that will enable ERα and ERβ specific functions to be investigated.

Lastly, in part, because the SKF-82958 experiments revealed that small molecules that have differential effects on ERα and ERβ transcriptional activity an be acting as agonists, we have also established a collaboration with Drs. Austin Cooney and Fernando Larrea. We have examined the ability of some A-ring reduced metabolites of 19-nor synthetic progestins (norethindrone and Gestodene) to regulate ERα and ERβ activity. We found that the 3β,5α derivatives of these compounds have a preferential ability to bind to (**Figure 14**) as well as activate ERα versus ERβ (**Figure 15**). This indicates that not only are there differences in how ligand-independent pathways regulate ER transcriptional activity, but also how ligands interact with and stimulate the activity of ERs.

Key Research Accomplishments

1. The oxytocin gene represents a promoter that can be regulated by ERα stimulated with forskolin/IBMX, but not ERβ.

2. The amino-terminal ERα phosphorylation sites are not required for forskolin/IBMX activation of ERα transcriptional activity.

3. AP-1 factors are able to interact with the TRE site previously demonstrated to be important for forskolin/IBMX activation of ERα and ERβ

4. Overexpression of c-Jun, but not c-Fos, contributes to forskolin/IBMX induction of ERα transcriptional activity.

5. The putative c-Jun and steroid receptor coactivator JAB1 does not strongly enhance the activity of either ERα or ERβ under conditions in which forskolin/IBMX can stimulate their transcriptional activity.

6. Cyclin D1 overexpression does not enhance ER transcriptional activity

7. A fragment of CBP that should block interaction of this coactivator with c-Jun blocks ERα and ERβ transcriptional activity.

8. All SRC family coactivators, as well as CBP, can coactivate ERα and ERβ transcriptional activity stimulated by forskolin/IBMX or E2

9. Phosphorylation of SRC-1 is not required for its ability to coactivate ERα activity stimulated by forskolin/IBMX

10. SKF-82958 is an agonist of ERα but not ERβ transcriptional activity.

11. SKF-82958 inhibits binding of estradiol to both ERα and ERβ.

12. 3β,5α-terahydro-derivatives of norethisterone and Gestodene are preferential ligands and agonists of ERα.

Reportable Outcomes

1. Larrea F, García-Becerra R, Lemus AE, García GA, Grillasca I, Pérez-Palacios G, Jackson K, Smith CL and Cooney AJ (2001): A-ring reduced metabolites of 19-nor synthetic progestins as selective agonists for estrogen receptor-α. *Endocrinology* 142:3791-3799.

2. Coleman KM and Smith CL (2001): Intracellular signaling pathways: non-genomic actions of estrogens and ligand-independent activation of estrogen receptors. *Frontiers in Bioscience,* in press.

3. Walters MR, Dutertre M and Smith CL: SKF-82958 is a subtype-selective estrogen receptor-α (ERα) agonist that induces ERα phosphorylation and synergistic functional interactions between ERα and AP-1. Submitted, 2001.

4. Coleman KM and Smith CL: Mechanistic differences in the activation of ERα- and ERβ-dependent gene expression by cAMP signaling pathway(s). The Endocrine Society – 83rd Annual Meeting, Denver, CO. Abstract #OR55-3; June 20-23, 2001.

The latter abstract is in preparation for publication as a portion of a manuscript tentatively entitled "Mechanistic differences in the activation of ERα- and ERβ-dependent gene expression by cAMP signaling pathway(s)" by Coleman KM, Dutertre M and Smith CL. Copies of items 1-4 can be found in the Appendix.

Conclusions

The originally published amino acid sequence of ERβ represents an amino-terminally truncated form, which lacks the first 45 amino acids of this receptor subtype. In transient transfection assays, ERα is clearly more active than the long and short forms of ERβ. The potentially large differences in ERβ$_S$ and ERβ$_L$ expression levels indicate that their relative expression levels must be taken into account when considering transactivation activity. Furthermore, the AF-1 activity of ERα exceeds that of ERβ and this likely contributes to the relative differences in transcriptional activity observed for these two receptor isotypes.

Both isotypes of estrogen receptor (α and β) can be activated in the absence of exogenous estrogens. In cells treated with forskolin and IBMX, ERα and were activated by a cAMP signaling pathway. This indicates that there is sufficient homology between these two receptor isotypes to mediate activation of gene expression by this signaling pathway. This activation pathway required the expression of estrogen receptors within the target cell, the presence of an estrogen response element in the target gene, and that the receptor can bind to DNA. However, the stimulation of receptor-dependent transcription can be significantly enhanced by the presence of the binding site for another transcription factor, in these studies an putative AP-1 binding site. Furthermore, the ability of ERα and ERβ to participate in this combinatorial response differs, supporting our original hypothesis that the ability of both estrogen receptor isotypes needs to be examined in order to determine the potential of each of these receptors to respond to ligand-independent signaling pathways.

We have investigated the ability of a number of coactivator proteins to stimulate ERα and ERβ transcriptional activity, and found that their potential to do so is variable. All SRC family members can coactivate ERα and ERβ transcriptional activity stimulated by forskolin/IBMX. This is consistent with their ability to interact with the receptor carboxy-termini, and the previously reported requirement of this domain for cAMP activation of transcription. In addition, CBP can also coactivate both forms of ER. These molecules are therefore are prime candidates to examine the relationship between ER and c-Jun with respect to cAMP activation of gene expression. Intriguingly, cAMP activation of PR and ERα also appears to be quite distinct. The former is not phosphorylated in response to cAMP signaling and appears to rely on modification to SRC-1. In contrast, ERα is phosphorylated and does not required SRC-1 phosphorylation to being about cAMP activation of gene expression. Thus, this pathway can communicate to different members of the steroid receptor superfamily via distinct signaling pathways. This is important because it implies that inhibition of cAMP activation of steroid receptor transcriptional activity may need to inhibit multiple molecule events to be achieved.

As anticipated, the experiments performed to date have provided information on the transcriptional activity of ERβ relative to ERα, as well as the ability of ERβ to respond to an alternative signaling pathway, induced by elevated intracellular cAMP, in the absence of estrogens. Taken together, this information will increase our understanding of the molecular

mechanisms by which ERα and ERβ respond to cross-talk pathways within a cell. It also will provide a framework for critical evaluation of whether it is possible to selectively regulate ERα and ERβ transcriptional activity.

Personnel Receiving Salary Support from this Award

Carolyn L. Smith

Kevin Coleman

Vinh Lam

Kate Ramsayer

Ratna Mukhopadhyay

References

1. Cullen, K. J. and Lippman, M. E. (1989) *Vitamins and Hormones* **45,** 127-172

2. Engel, L. W. and Young, N. A. (1978) *Cancer Res.* **38,** 4327-4339

3. Evans, R. M. (1988) *Science* **240,** 889-895

4. Tsai, M.-J. and O'Malley, B. W. (1994) *Ann.Rev.Biochem.* **63,** 451-486

5. Tsai, S. Y., Carlstedt-Duke, J., Weigel, N. L., Dahlman, K., Gustafsson, J.-A., Tsai, M.-J., and O'Malley, B. W. (1988) *Cell* **55,** 361-369

6. Kumar, V. and Chambon, P. (1988) *Cell* **55,** 145-156

7. Aronica, S. M. and Katzenellenbogen, B. S. (1993) *Mol.Endocrinol.* **7,** 743-752

8. Denton, R. R., Koszewski, N. J., and Notides, A. C. (1992) *J.Biol.Chem.* **267(11),** 7263-7268

9. Washburn, T. F., Hocutt, A., Brautigan, D. L., and Korach, K. S. (1991) *Mol Endocrinol* **5,** 235-242

10. Ali, S., Metzger, D., Bornert, J. M., and Chambon, P. (1993) *EMBO J.* **12(3),** 1153-1160

11. Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. (1994) *Mol.Endocrinol.* **8,** 1208-1214

12. Goff, P. L., Montano, M. M., Schodin, D. J., and Katzenellenbogen, B. S. (1994) *J.Biol.Chem.* **269,** 4458-4466

13. Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.-R., and Chambon, P. (1987) *Cell* **51,** 941-951

14. Webster, N. J. G., Green, S., Jin, J.-R., and Chambon, P. (1988) *Cell* **54,** 199-207

15. Webster, N. J. G., Green, S., Tasset, D., Ponglikitmongkol, M., and Chambon, P. (1989) *EMBO J.* **8,** 1441-1446

16. Fawell, S. E., Lees, J. A., White, R., and Parker, M. G. (1990) *Cell* **60,** 953-962

17. Smith, C. L., Conneely, O. M., and O'Malley, B. W. (1993) *Proc.Natl.Acad.Sci.USA* **90,** 6120-6124

18. Ignar-Trowbridge, D. M., Pimentel, M., Parker, M. G., McLachlan, J. A., and Korach, K. S. (1996) *Endocrinology* **137,** 1735-1744

19. Power, R. F., Mani, S. K., Codina, J., Conneely, O. M., and O'Malley, B. W. (1991) *Science* **254,** 1636-1639

20. Newton, C. J., Buric, R., Trapp, T., Brockmeier, S., Pagotto, U., and Stalla, G. K. (1994) *J.Steroid Biochem.Molec.Biol.* **48,** 481-486

21. Ma, Z. Q., Santagati, S., Patrone, C., Pollio, G., Vegeto, E., and Maggi, A. (1994) *Mol.Endocrinol.* **8,** 910-918

22. Ignar-Trowbridge, D. M., Teng, C. T., Ross, K. A., Parker, M. G., Korach, K. S., and McLachlan, J. A. (1993) *Mol.Endocrinol.* **7,** 992-998

23. Pietras, R. J., Arboleda, J., Reese, D. M., Wongvipat, N., Pegram, M. D., Ramos, L., Gorman, C. M., Parker, M. G., Sliwkowski, M. X., and Slamon, D. J. (1995) *Oncogene* **10,** 2435-2446

24. Zwijsen, R. M. L., Wientjens, E., Klompmaker, R., van der Sman, J., Bernards, R., and Michalides, R. J. A. M. (1997) *Cell* **88,** 405-415

25. Curtis, S. W., Washburn, T. F., Sewall, C., DiAugustine, R., Lindzey, J., Couse, J. F., and Korach, K. (1996) *Proc.Natl.Acad.Sci.USA* **93,** 12626-12630

26. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) *Science* **270,** 1491-1494

27. Joel, P. B., Traish, A. M., and Lannigan, D. A. (1995) *Mol.Endocrinol.* **9,** 1041-1052

28. Bunone, G., Briand, P.-A., Miksicek, R. J., and Picard, D. (1996) *The EMBO Journal* **15,** 2174-2183

29. El-Tanani, M. and Green, C. D. (1997) *Mol.Endocrinol.* **11,** 928-937

30. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. (1996) *Proc.Natl.Acad.Sci.USA* **93,** 5925-5930

31. Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J. (1997) *Endocrinology* **138,** 863-870

32. Tremblay, G. B., Tremblay, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Labrie, F., and Giguere, V. (1997) *Mol.Endocrinol.* **11,** 353-365

33. Korach, K. S., Couse, J. F., Curtis, S. W., Washburn, T. F., Linzey, J., Kimbro, K. S., Eddy, E. M., Migliaccio, S., Snedeker, S. M., Lubahn, D. B., Schomberg, D. W., and Smith, E. P. (1996) *Recent Progress in Hormone Research* **51,** 159-188

34. Mosselman, S., Polman, J., and Dijkema, R. (1996) *FEBS Letters* **392,** 49-53

35. Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantx, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J.-A. (1997) *J.Clin.Endocrinol.Metab.* **82,** 4258-4265

36. Lu, B., Leygue, E., Dotzlaw, H., Murphy, L. J., Murphy, L. C., and Watson, P. H. (1998) *Mol and Cell Endocrin* **138,** 199-203

37. Jonat, G., Rahmsdorf, H. J., Park, K. K., Cato, A. C. B., Gebel, S., Ponta, H., and Herrlich, P. (1990) *Cell* **62,** 1189-1204

38. de Groot, R. P. and Sassone-Corsi, P. (1992) *Oncogene* **7,** 2281-2286

39. Kvanta, A. and Fredholm, B. B. (1993) *J.Mol.Neurosci.* **4,** 205-214

40. Aronica, S. M. and Katzenellenbogen, B. S. (1991) *Endocrinol.* **128,** 2045-2052

41. Sumida, C. and Pasqualini, J. R. (1990) *Mol.Cell Endocrinol.* **69,** 207-215

42. Webb, P., Lopez, G. N., Uht, R. M., and Kushner, P. J. (1995) *Mol.Endocrinol.* **9,** 443-456

43. Hanstein, B., Eckner, R., DiRenzo, J., Halachmi, S., Liu, H., Searcy, B., Kurokawa, R., and Brown, M. (1996) *Proc.Natl.Acad.Sci.USA* **93,** 11540-11545

44. Arias, J., Alberts, A. S., Brindle, P., Claret, F. X., Smeal, T., Karin, M., Feramisco, J., and Montminy, M. (1994) *Nature* **370,** 226-229

45. Bannister, A. J., Oehler, T., Wilhelm, D., Angel, P., and Kouzarides, T. (1995) *Oncogene* **11,** 2509-2514

46. LeGoff, P., Montano, M. M., Schodin, D. J., and Katzenellenbogen, B. S. (1994) *J.Biol.Chem.* **269(6),** 4458-4466

47. Karin, M., Liu, Z., and Zandi, E. (1997) *Current Opinion in Cell Biology* **9,** 240-246

48. Chauchereau, A., Georgiakaki, M., Perrin-Wolff, M., Milgrom, E., and Loosfelt, H. (2000) *J.Biol.Chem.* **275,** 8540-8548

49. McMahon, C., Suthiphongchai, T., DiRenzo, J., and Ewen, M. E. (1999) *Proceedings of the National Academy of Sciences of the USA* **96,** 5382-5387

50. Zwijsen, R. M., Buckle, R. S., Hijmans, E. M., Loomans, C. J., and Bernards, R. (1998) *Genes and Dev.* **12,** 3488-3498

51. Lamb, J., Ladha, M. H., McMahon, C., Sutherland, R. L., and Ewen, M. E. (2000) *Mol.Cell.Biol.* **20,** 8667-8675

52. Smith, C. L., Onate, S. A., Tsai, M.-J., and O'Malley, B. W. (1996) *Proc Natl Acad Sci USA* **93,** 8884-8888

53. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) *Cell* **85,** 403-414

54. Smith, C. L., Nawaz, Z., and O'Malley, B. W. (1997) *Mol.Endocrinol.* **11,** 657-666

55. Rowan, B. G., Garrison, N., Weigel, N. L., and O'Malley, B. W. (2000) *Mol Cell Biol* **20,** 8720-8730

56. Gangolli, E. A., Conneely, O. M., and O'Malley, B. W. (1997) *J.Steroid Biochem.Molec.Biol.* **61,** 1-9

57. Lonard, D. M., Nawaz, Z., Smith, C. L., and O'Malley, B. W. (2000) *Molecular Cell* **5,** 939-948

58. O'Boyle, K. M., Gaitanopoulos, D. E., Brenner, M., and Waddington, J. L. (1989) *Neuropharmacology* **28,** 401-405

59. Kurten, R. C. and Richards, J. S. (1989) *Endocrinology* **125,** 1345-1357

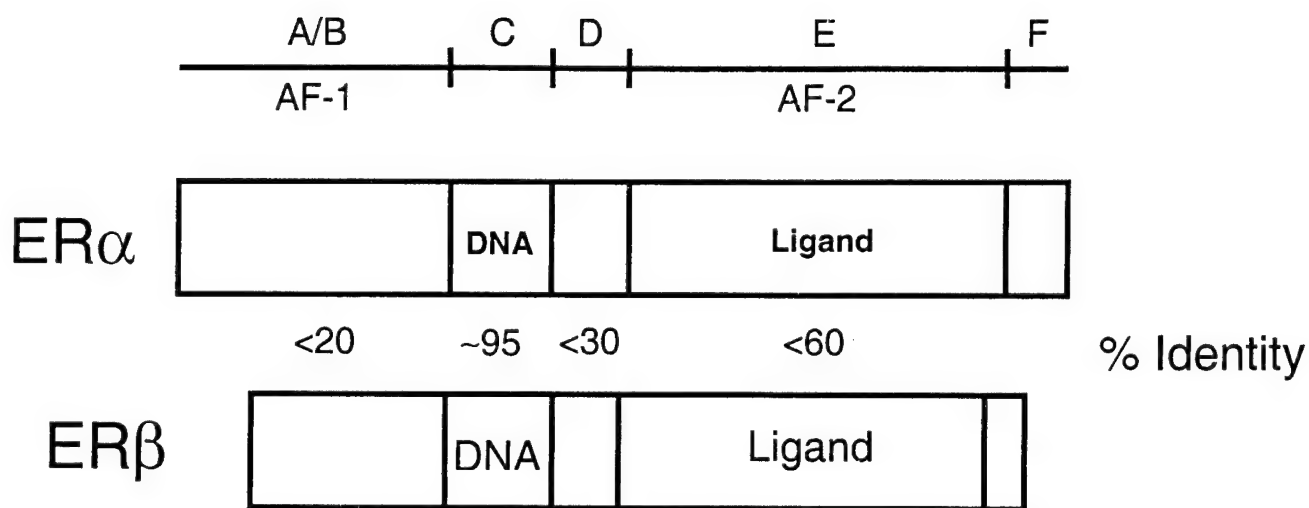60. Jordan, V. C. (1984) *Pharmacol.Rev.* **36,** 245-276

Appendices

Figure 1: Structure of ERα and ERβ. The *top panel* represents the location of various regions of estrogen receptor (A to F) and its activation functions (AF-1 and AF-2). The bottom panel represents the comparative structure of ERα and ERβ. Values given between the two receptor forms represent approximately homologies in their respective amino acid sequences.
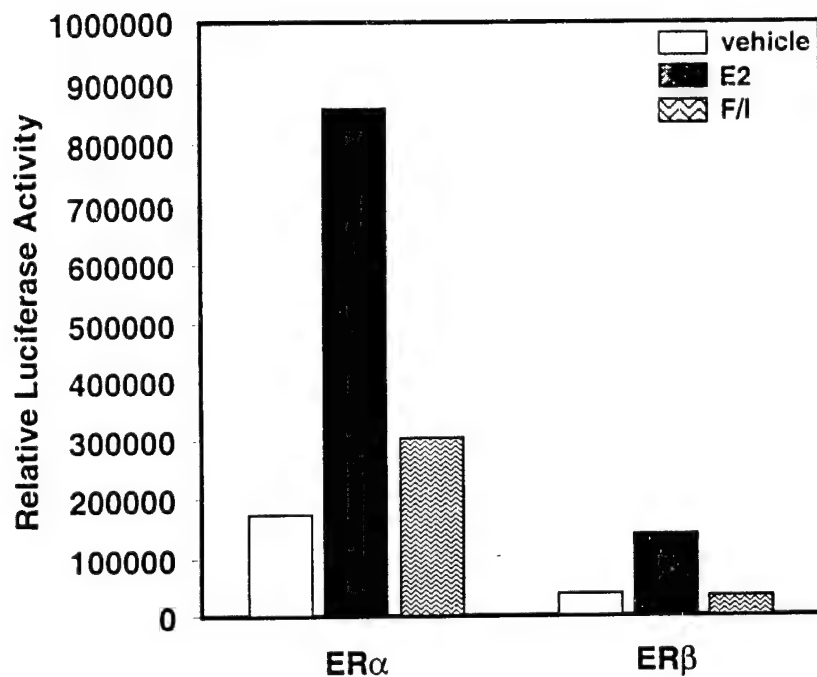
Figure 2: Forskolin stimulates ERα- but not ERβ-dependent transcription of the oxytocin promoter. HeLa cells were transfected with 1 μg Ro-Luc reporter along with 10 ng pCMV$_5$-ERα or pCXN$_2$-ERβ. Cells were treated for 24 hours with EtOH (vehicle), 1 nM estradiol (E2) or 10 μM forskolin and 100 μM IBMX (F/I) and luciferase activity was measured. The graph is a representative experiment.
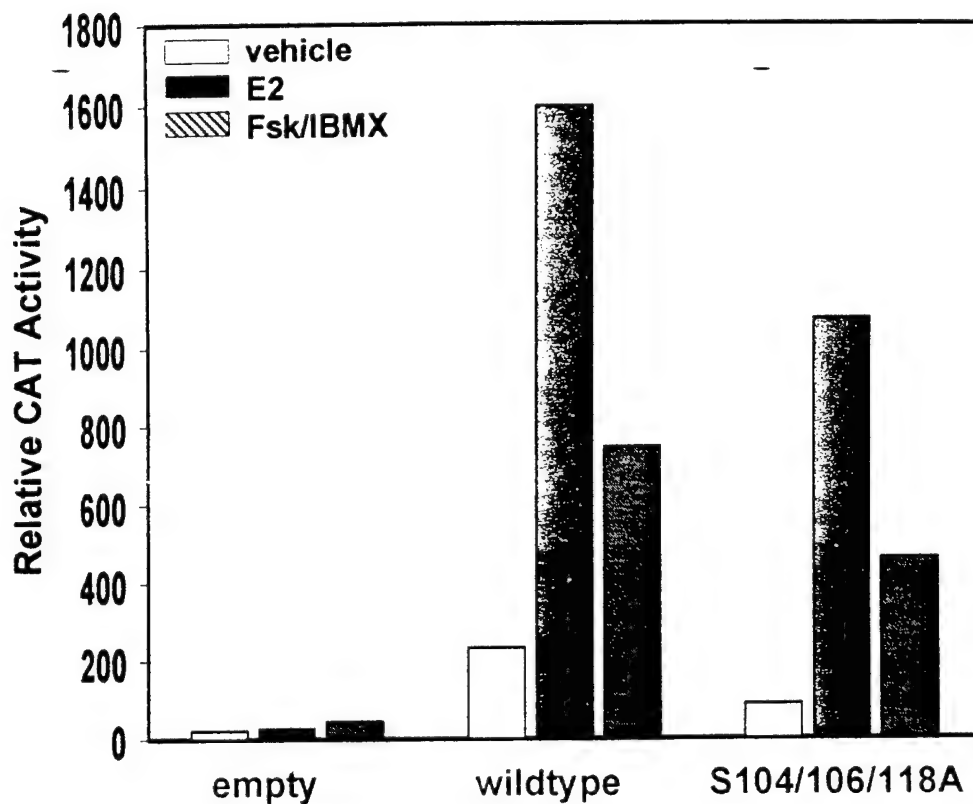
Figure 3: Forskolin stimulates transcriptional activity of a ERα phophorylation sites mutant. HeLa cells were transfected with 1 μg mutated ERE-E1b-CAT (mTRE) along with 10 ng pCMV₅-ERα, pCMV₅-ERα-S104/106/167A or empty vector control. Cells were treated with EtOH, 1 nM estradiol (E2) or 10 μM forskolin + 100 μM IBMX (F/I). CAT activity was measured and normalized to total protein. The graph is a representative experiment.
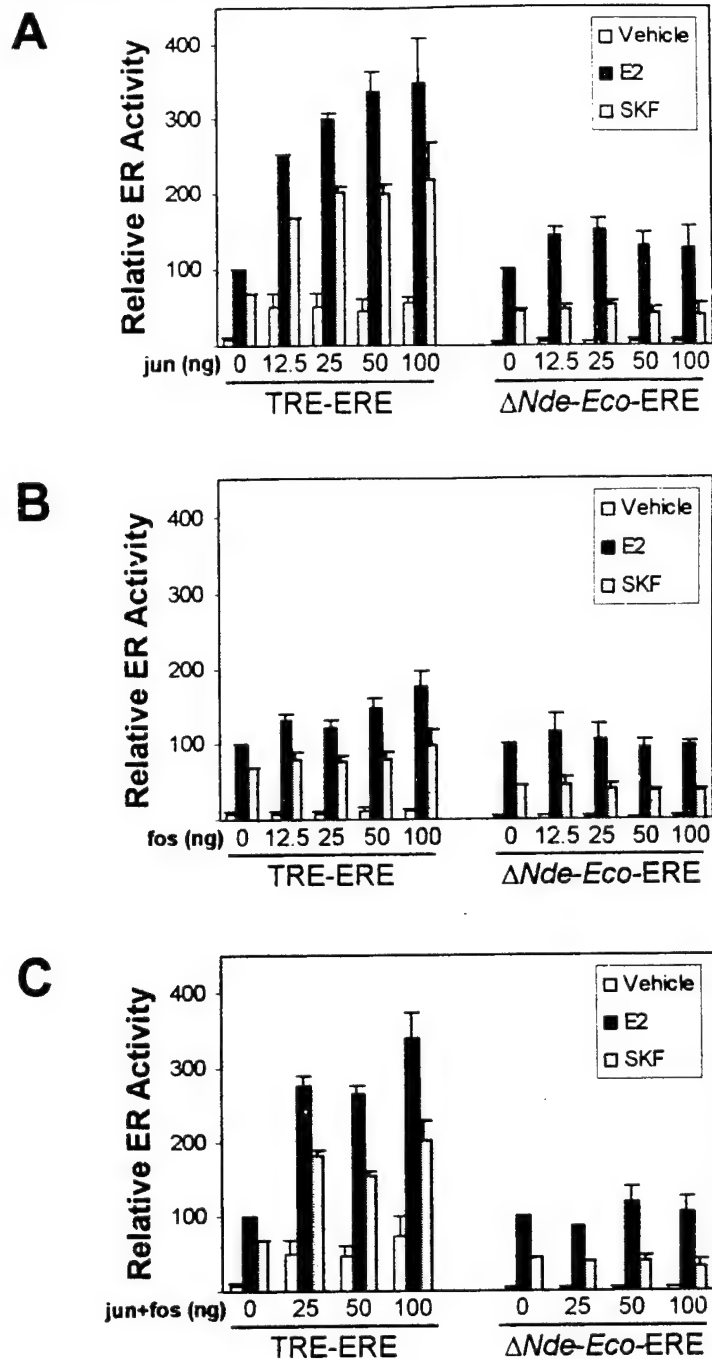
Figure 4: Overexpression of c-Jun enhances ERα transcriptional activity. HeLa cells were co-transfected with increasing concentrations of expression plasmid for **(A)** c-Jun **(B)** c-Fos or **(C)** equivalent amounts of c-Jun and c-Fos along with pSVMT-wtER and ERE-E1b-CAT reporter genes with (TRE-ERE) or without (Δ*Nde1-Eco*-ERE) a TRE. Total DNA levels were normalized in each group by co-transfecting appropriate levels of the empty plasmid pRSV-Not. Transfections were done 6h prior to addition of the indicated agonists, with harvest following 18h thereafter. Cells were treated with ethanol (vehicle), 1 nM E2 or 10 μM SKF-82958 (SKF). Bars represent mean ± SEM for n=3 independent experiments and values are expressed relative to the CAT activity (100) induced by E2 treatment from ERE-E1b-CAT in each experiment.
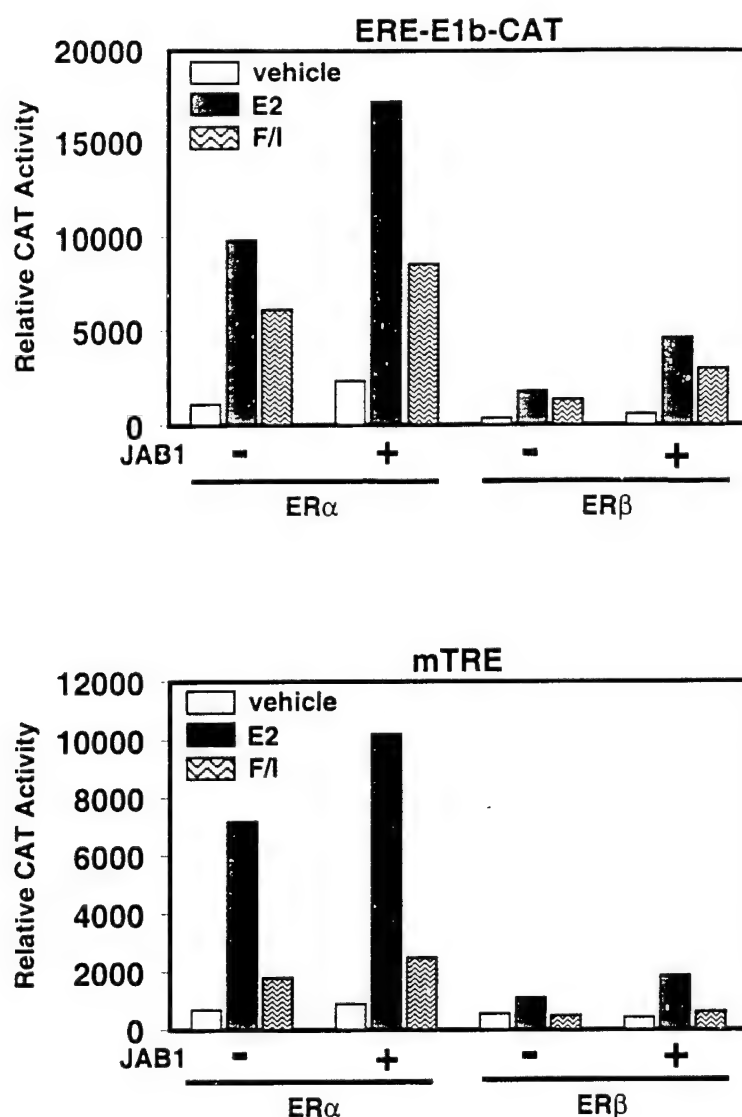
Figure 5: cAMP-dependent ER activity is not significantly altered by JAB1 coactivator.
HeLa cells were transfected with 750 ng ERE-E1b-CAT or the mutated ERE-E1b-CAT (mTRE)
along with 25 ng $pCMV_5$-ERα or $pCXN_2$-ERβ and 500 ng pCR3.1-JAB1 or vector control.
Cells were treated for 24 hours with EtOH (vehicle), 1 nM estradiol (E2) or 10 μM forskolin and
100 μM IBMX (F/I) and luciferase activity was measured. The graph is a representative
experiment.

Figure 6: Cyclin D1 inhibits ER Transactivation. HeLa cells were transfected with 750 ng ERE-E1b-CAT reporter along with 25 ng pCMV$_5$-ERα or pCXN2-ERβ and either 1 µg pCMV$_5$-Cyclin D1 or vector control. Cells were treated for 24hours with EtOH, 1nM estradiol (E2) or 10 µM forskolin plus 100µM IBMX (F/I) and luciferase activity was measured. The graph is a representative experiment.

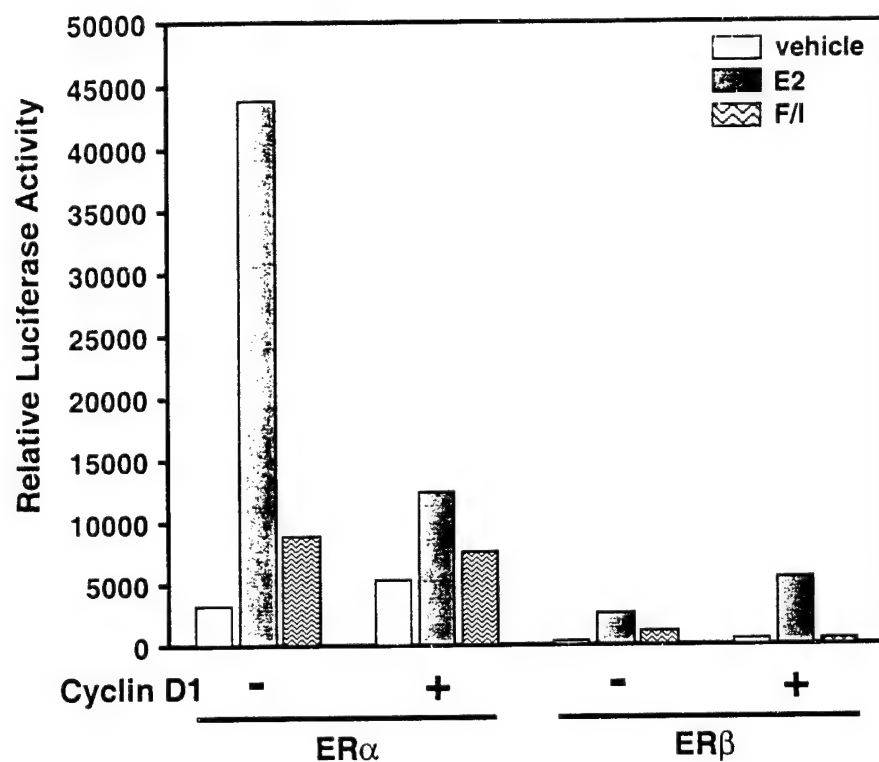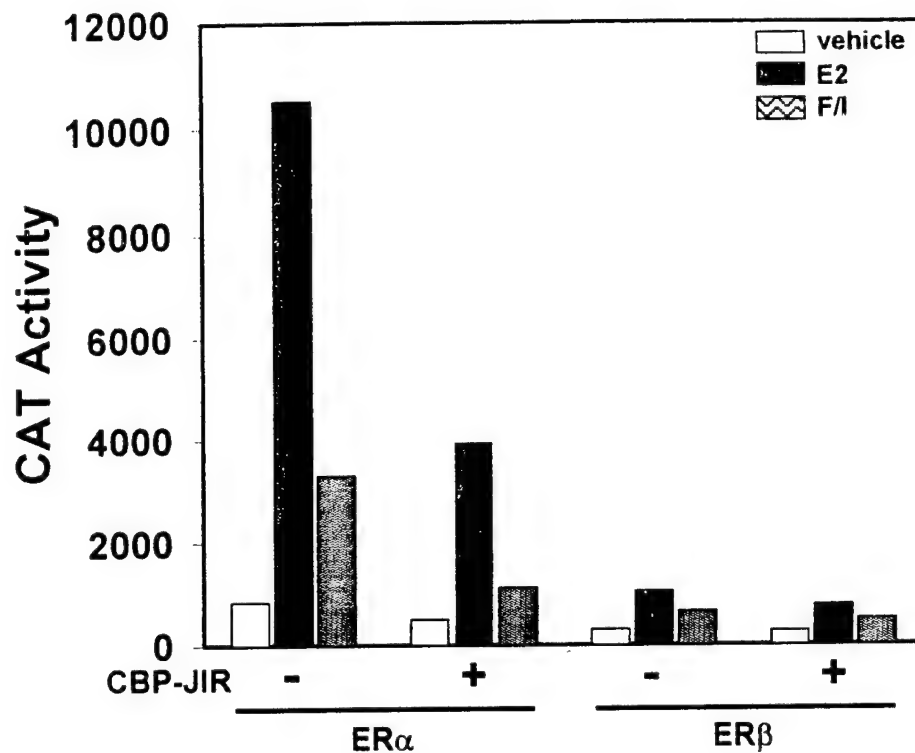**Figure 7: CBP-JIR decreases hormone-dependent and hormone-independent transcriptional activity.** HeLa cells were transfected with 500 ng ERE-E1b-CAT along with 25 ng of pCMV$_5$-ERα or pCXN$_2$-ERβ and 750 ng of pCR3.1-CBP-JIR or empty vector control. Cells were treated with EtOH, 1nM estradiol (E2) or 10 μM forskolin + 100 μM IBMX (F/I). CAT activity was measured and normalized to total protein. The graph is a representative experiment.
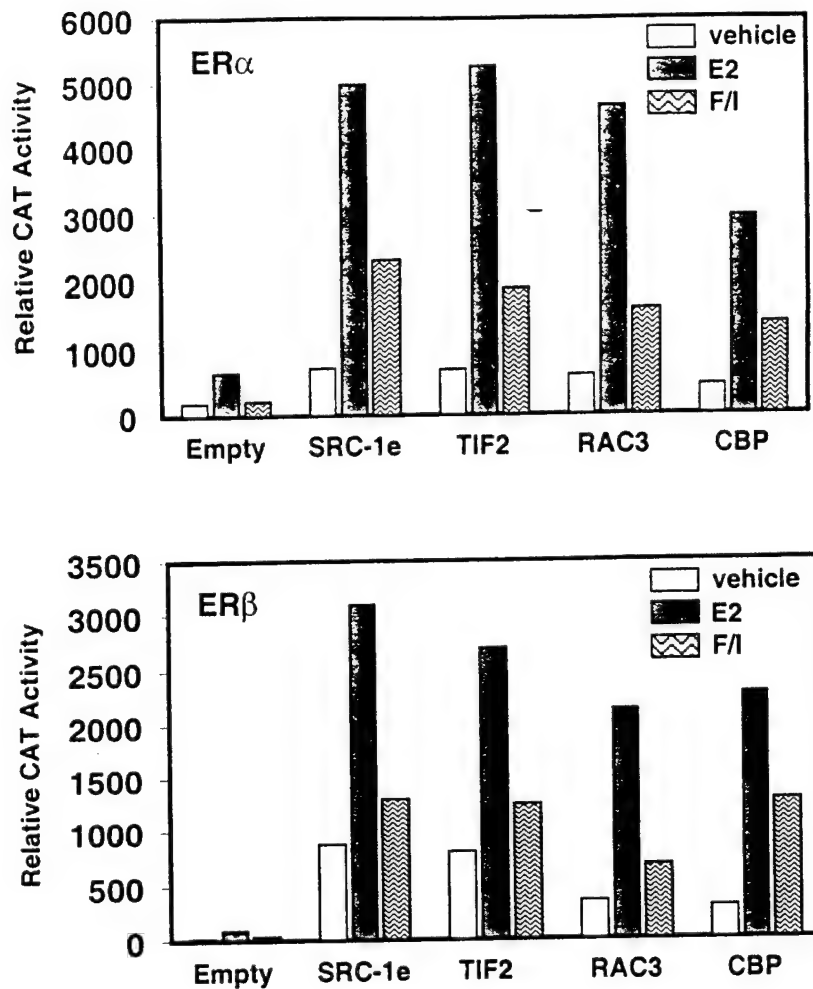
Figure 8: Enhancement of cAMP-dependent ER activity by SRC family and CBP coactivators. HeLa cells were transfected with 750 ng ERE-E1b-CAT along with 25 ng pCMV$_5$-ERα or pCXN$_2$-ERβ and 1 μg pCR3.1-SRC-1e, pCR3.1-TIF2, pCR3.1-RAC3, pCR3.1-CBP or vector control and 100 ng β-gal (top). Cells were treated for 24 hours with EtOH, 1nM estradiol (E2) or 10 μM forskolin and 100 μM IBMX (F/I). CAT activity was measured and normalized to β-gal activity (top) or total protein (bottom). The graphs are representative experiments.
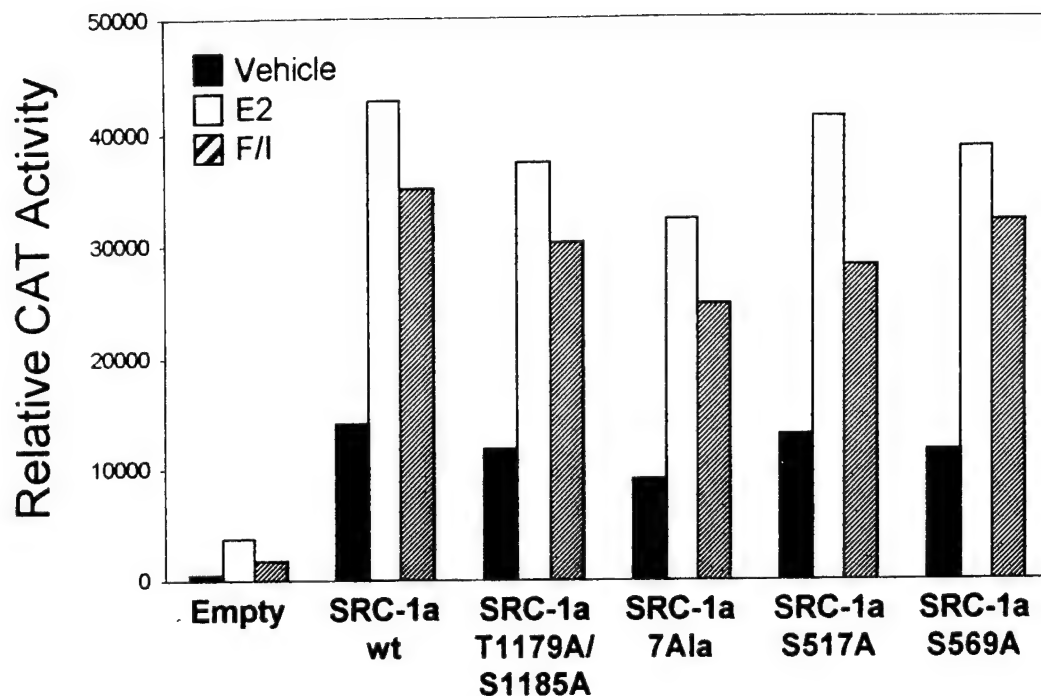
Figure 9: Influence of SRC-1 phosphorylation site mutations on coactivation of ERα transcriptional activity. HeLa cells were transfected with 1 μg ERE-E1b-CAT along with 10 ng pCMV$_5$-ERα and 1 μg of wild type (pCR3.1-SRC-1a) or mutant forms of SRC-1. Cells were treated for 24 hours with EtOH, 1nM estradiol (E2) or 10 μM forskolin and 100 μM IBMX (F/I). CAT activity was measured and normalized to total protein. The graph is a representative experiment.
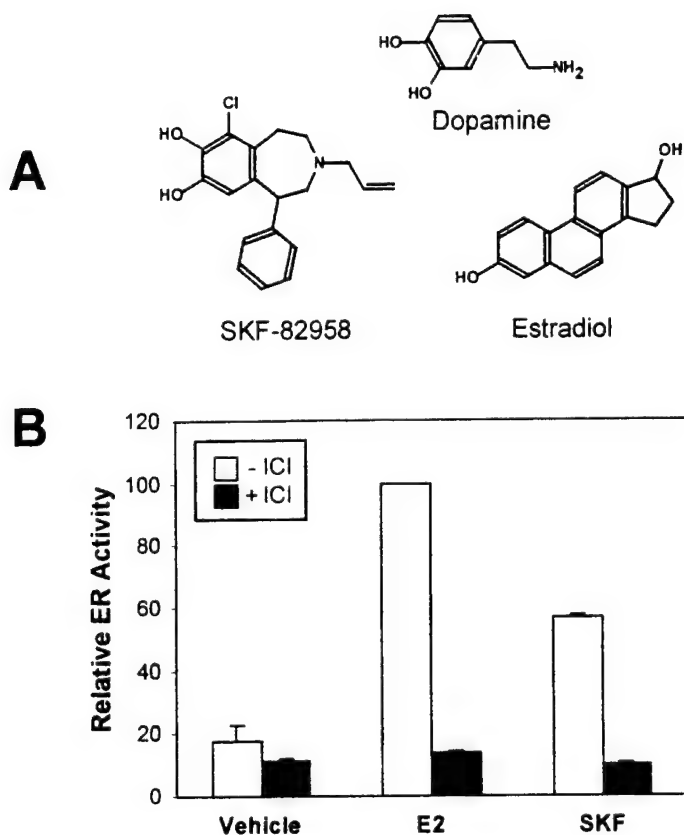
Figure 10: SKF-82958 activates ERα-dependent gene expression. (A) Chemical structures of the compounds used to regulate ERα activity. (B) Activation of ERE-E1b-Luc target gene expression by SKF-82958 is receptor dependent. HeLa cells were transfected with expression vectors for ERα (pCMV5-hERβ) and β-galactosidase (pCMVβ), and the ERE-E1b-Luc reporter gene and subsequently treated with ethanol (vehicle), 1 nM E2 or 10 μM SKF-82958 (SKF) in the absence or presence of 100 nM ICI 182,780. Data represent the average of three independent experiments ± SEM.

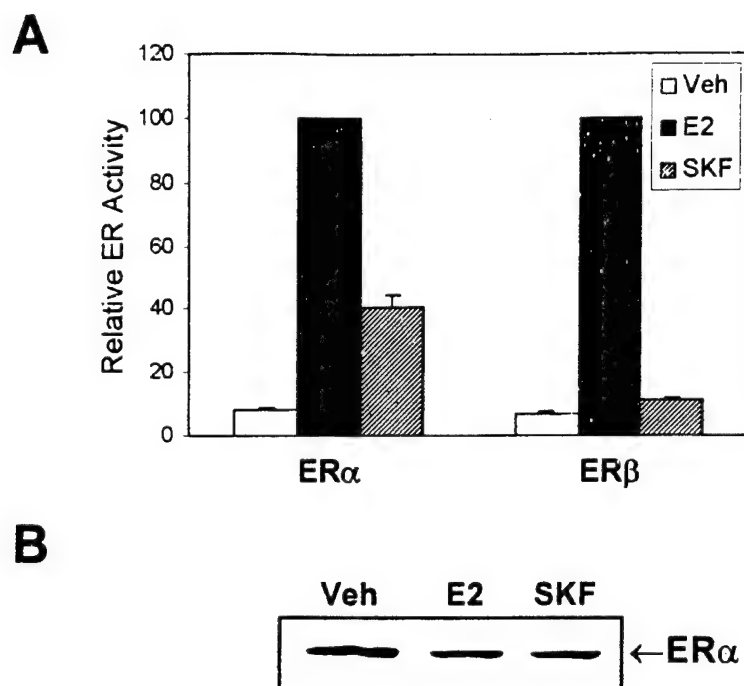**Figure 11: SKF-82958 is an ERα selective activator of transcription.** (A) HeLa cells were transfected with expression vectors for ERα (pCMV₅-hERα) or ERβ (pCMV₅-hERβ) along with ERE-E1b-Luc and pCMVβ, and subsequently treated with ethanol (Veh), 1 nM E2 or 10 μM SKF-82958. Data represent the average of four independent experiments ± SEM.

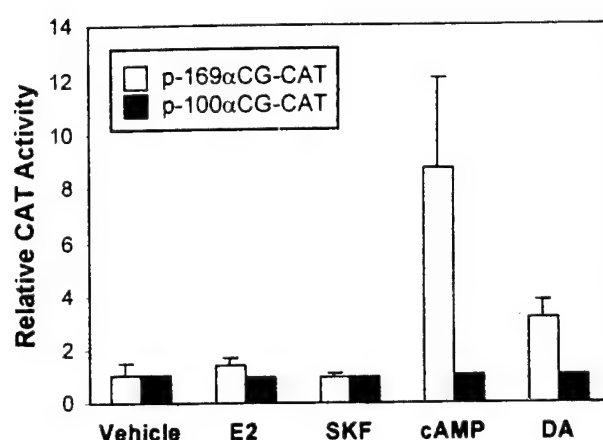**Figure 12: SKF-82958 does not stimulate CREB transcriptional activity.** HeLa cells were transfected with either a CRE-containing (p-169αCG-CAT) or CRE-minus (p-100αCG-CAT) reporter gene and subsequently treated with ethanol (Vehicle), 1 nM E2, 25 μM SKF-82958 (SKF), 1 mM 8Br-cAMP and 100 μM IBMX (cAMP) or 200 μM dopamine (DA). CREB activation data represent the average of three independent experiments ± SEM.
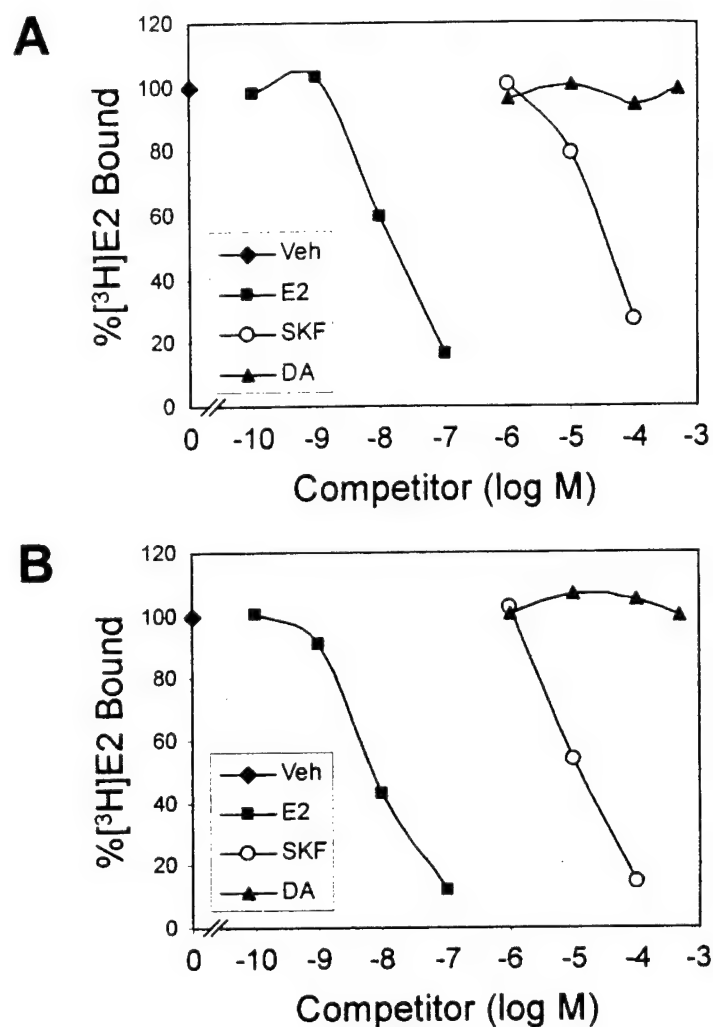
Figure 13: SKF-82958 binds to ERα and ERβ. *In vivo* hormone binding assays of (**A**) ERα or (**B**) ERβ were performed to assess the relative binding affinity of E2, SKF-82958 (SKF) or dopamine (DA) with respect to competition for [³H]estradiol binding to receptor. Total [³H]estradiol binding in the absence of competitor (♦) is shown for cells treated with ethanol (Veh). Values represent the average of duplicate samples from a representative experiment (n=3-4).
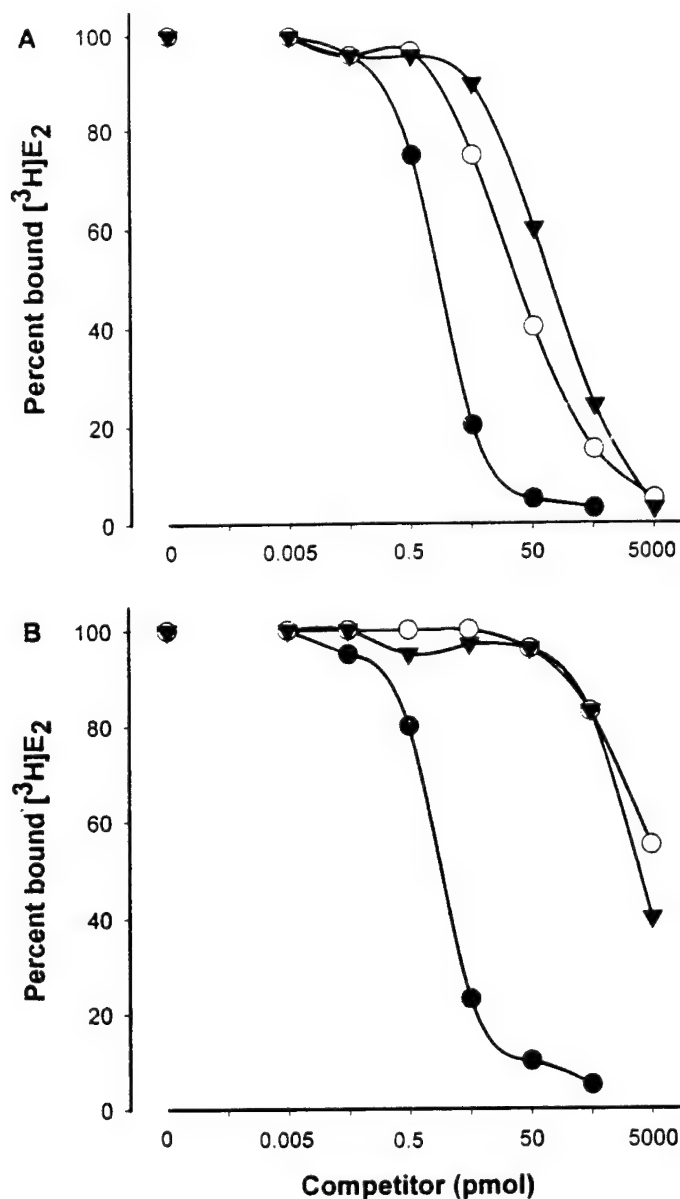
Figure 14: Relative binding affinity of E2 (closed circles), 3β,5α-NET (open circles) and 3β,5α-GSD (triangle) for ERα (A) and ERβ (B). Extracts from COS-1 cells transfected with expression vectors for either ERα or ERβ were incubated in the presence of 1 pmol [³H]E2 and increasing concentrations (0.005-5000 pmol) of each of the unlabeled competitors. Free from receptor-bound steroid was separated by adsorption to hydroxyapatite. Values are the mean of a representative experiment performed in duplicate.
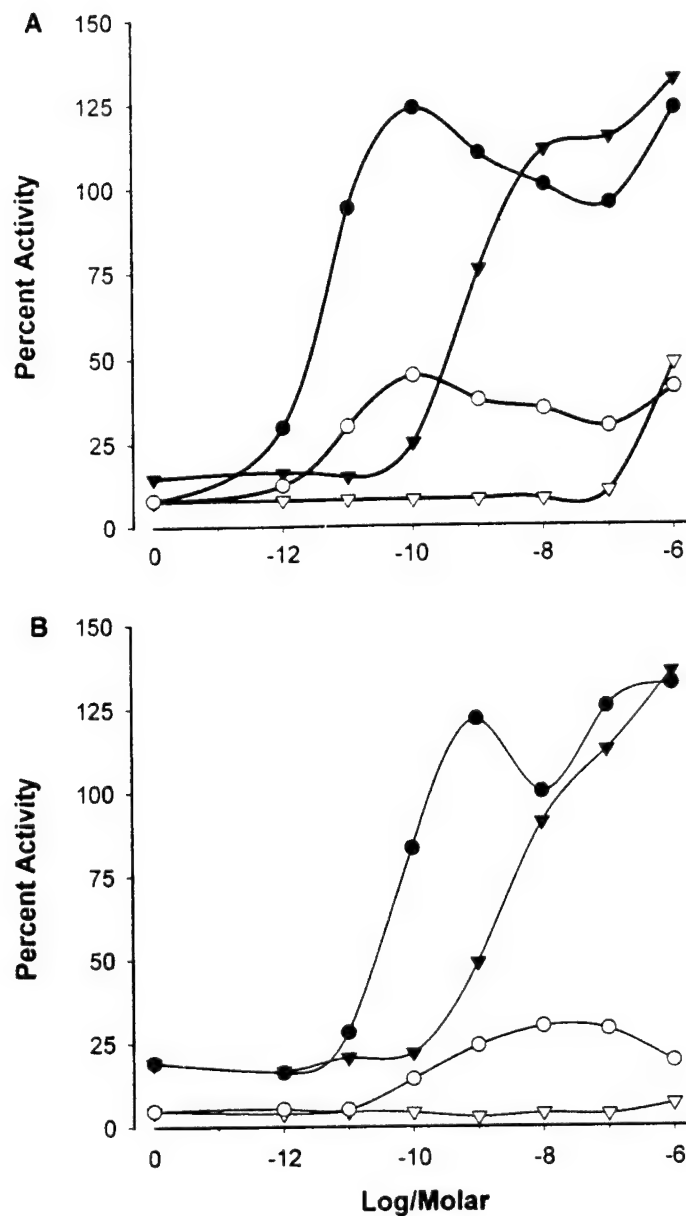
Figure 15: Dose-dependent activation of ERα (closed symbols) but not ERβ (open symbols) by 3β,5α-NET (A) and 3β,5α-GSD (B). HeLa cells were transiently transfected with expression vectors for ERα (filled symbols) or ERβ (open symbol) and an ERE-E1b-CAT reporter gene and cultured in the absence or presence of increasing concentrations (10^{-12} to 10^{-6} M) of E2 (circles) or the 3β,5α derivative (triangles) of NET and GSD, respectively. Values are the mean ± SD of triplicate experiments. The data are represented as percent activity relative to values obtained for ERα treated with 10^{-8} M E2.

A-Ring Reduced Metabolites of 19-nor Synthetic Progestins as Subtype Selective Agonists for ERα

FERNANDO LARREA, ROCIO GARCíA-BECERRA, ANA E. LEMUS, GUSTAVO A. GARCíA, GREGORIO PÉREZ-PALACIOS, KATHY J. JACKSON, KEVIN M. COLEMAN, ROSLYN DACE, CAROLYN L. SMITH, AND AUSTIN J. COONEY

Department of Reproductive Biology (F.L., R.G.-B.), Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico 14000; Department of Reproductive Biology (A.E.L.), Universidad Autónoma Metropolitana-Iztapalapa, Mexico City, Mexico 09340; Facultad de Química (G.A.G.), Universidad Nacional Autónoma de México, Mexico City, Mexico 04510; School of Medicine (G.P.-P.), Universidad Nacional Autónoma de México, Mexico City General Hospital, Mexico 06726; and Department of Molecular and Cellular Biology (K.J.J., K.M.C., R.D., C.L.S., A.J.C.), Baylor College of Medicine, Houston, Texas 77030

It has previously been demonstrated that 19-nor contraceptive progestins undergo *in vivo* and *in vitro* enzyme-mediated A-ring double bond hydrogenation. Bioconversion of 19-nor progestins to their corresponding tetrahydro derivatives results in the loss of progestational activity and acquisition of estrogenic activities and binding to the ER. Herein, we report subtype-selective differences in ligand binding and transcriptional potency of nonphenolic synthetic 19-nor derivatives between ERα and ERβ. In this study, we have examined both ER- and PR-mediated transcriptional activity of a number of A-ring chemically reduced derivatives of norethisterone and Gestodene. Double bond hydrogenation decreased the transcriptional potency of norethisterone and Gestodene through both PR isoforms with a 100- to 1,000-fold difference, respectively. In terms of the effects of norethisterone and Gestodene and their corresponding 5α-dihydro (5α-norethisterone and 5α-Gestodene), or 3α,5α-tetrahydro or 3β,5α-tetrahydro derivatives (3α,5α-norethisterone/3α,5α-Gestodene and 3β,5α-norethisterone/3β,5α-Gestodene, respectively) on estrogen-mediated transcriptional regulation, the 3β,5α-tetrahydro derivatives of both norethisterone and Gestodene showed the highest induction when HeLa cells were transiently transfected with an expression vector for ERα. This activity could be inhibited with tamoxifen. These compounds did not activate gene transcription via ERβ, and none of them showed antagonistic activities through either ER subtype. The 3β,5α-tetrahydro derivatives of both norethisterone and Gestodene were active in other cells in addition to HeLa cells and activated reporter expression through the oxytocin promoter. In summary, two ERα selective agonists have been identified. These compounds, with ERα vs. ERβ selective agonist activity, may be useful in evaluating the distinct role of these receptors as well as in providing useful insights into ER action. (**Endocrinology** 142: 3791–3799, 2001)

THE ER, WHICH is a member of the nuclear receptor superfamily, functions as a hormone-dependent transcription factor (1). Transactivation by the ER is mediated by two activation functions (AF): AF-1 is located in the N-terminal domain, and AF-2 is located in the C-terminal ligand-binding domain of the receptor (2). Recently, a novel ER isoform was identified that is preferentially expressed in human and rat ovary and prostate (3). This receptor has been named ERβ to distinguish it from the classical ERα. Although the biological significance of the existence of two ER subtypes is still unclear, the relative homology (60%) between their respective ligand binding domains (4) raises the possibility of the existence of natural or synthetic ligands with unique binding characteristics for ERα and ERβ subtypes. Thus, the differential expression and unique ligand binding specificities of the

ER subtypes could provide an explanation for the pleotropic actions of estrogens in many target tissues (5, 6). We, and others, have previously demonstrated that 19-nor progestins are bio-transformed into several metabolites that exhibit altered hormone properties in target tissues (7–10). A-ring reduction of 19-nor T derivatives such as norethisterone (NET), Gestodene (GSD), and levonorgestrel (LNG) to their corresponding 5α-dihydro and 3β,5α-tetrahydro metabolites significantly reduces their progestational activity. Although the dihydro-reduced metabolites bind mainly to PRs, the tetrahydro-reduced metabolites lose their progestational activity and demonstrate significant binding affinity for the ER with *in vivo* estrogenic effects (9–13). These observations support the idea that a given steroid can induce selective and even opposing effects in a variety of organs and tissues depending on its metabolic fate, the availability of steroid receptors, and the presence of different subsets of available steroid-responsive promoters and cofactors.

In the present study, we have evaluated the estrogenic activities of NET and GSD and their metabolites using transient transfections in HeLa and CHO cells with an estrogen

response element driven chloramphenicol acetyltransferase (CAT) reporter or the oxytocin luciferase reporter and expression vectors for either ERα or ERβ. The results demonstrated that at low concentrations the 3β,5α-tetrahydro derivatives of both NET and GSD (3β,5α-NET and 3β,5α-GSD, respectively) selectively activate ERα, whereas a weak ERβ agonistic activity was observed only with the 3β,5α-NET derivative at very high concentrations. It appears, therefore, that there are some unique features in the structure of these compounds that promote specific binding to ERα and transactivation via this receptor.

Materials and Methods

Reagents

Nonradioactive E2 was purchased from Sigma (St. Louis, MO). [2,4,6,7-³H]Estradiol ([³H]E2; specific activity 72 Ci/mmol) and [³H]chloramphenicol (specific activity 38.9 Ci/mmol) were purchased from NEN Life Science Products Research products (Boston, MA). Radioactivity was determined in a Beckman Coulter, Inc. LS6500 scintillation system (Beckman Coulter, Inc., Fullerton, CA) using Biodegradable Counting Scintillant (Amersham Pharmacia Biotech, Piscataway, NJ) as counting solution. Cell culture medium was purchased from Life Technologies, Inc. (Grand Island, NY). FBS was from HyClone Laboratories, Inc. (Logan, UT). All other solvents and reagents used were of analytical grade. Authentic NET (17α-ethynyl-17β-hydroxy-4-estren-3-one) and GSD (13β-ethyl-17α-ethynil-17β-hydroxy-4,15-gonadien-3-one) were kindly provided by Schering AG Mexicana, S.A. (Mexico City) and Schering AG (Berlin, Germany), respectively. Synthesis of the corresponding 5α-dihydro (5α-NET and 5α-GSD), and the 3α,5α- (3α,5α-NET and 3α,5α-GSD) and 3β,5α-(3β,5α-NET and 3β,5α-GSD) tetrahydro derivatives, including the description of their corresponding physical and spectroscopic constants has been previously described (12, 13).

Plasmids

The pLEN-hPR$_A$ was constructed by inserting the full-length human progesterone receptor (PR$_A$) cDNA into the *Bam*HI site of the pLEN mammalian expression vector (14). The pLEN-hPR$_B$ was generated by inserting the full-length human PR$_B$ cDNA into the *Bam*HI site of the pLEN vector. The expression vectors for human ERα and ERβ (pCMV$_5$-hERα and pCMV$_5$-hERβ) containing the coding sequence of the ERα and ERβ were kindly provided by Drs. B. S. Katzenellenbogen, University of Illinois (Urbana, IL) and J.-Å. Gustafsson, Karolinska Institute (Huddinge, Sweden), respectively. The estrogen responsive reporter plasmid (ERE-E1b-CAT) contains a fragment of the vitellogenin A2 gene promoter (positions −331 to −87) upstream of the adenovirus E1b TATA box fused to the chloramphenicol acetyltransferase (CAT) gene (15). The progesterone responsive reporter plasmid (PRE-E1b-CAT) was used as a reporter for PR$_A$ and PR$_B$ (16). The oxytocin reporter, pROLUC, has been previously described (17).

Transfections and reporter assays

HeLa and CHO cells were plated the day before transfections, at a density of 3.0 × 10⁵ cells/well/6-well plate, in DMEM without phenol red (DMEM-HG), which was supplemented with 5% stripped FBS and 100 U/ml of penicillin and 100 µg/ml streptomycin; and incubated in 5% CO₂ at 37 C. The next day, the HeLa or CHO cells were visualized on a microscope to verify that the cell density was 30–50% confluent. Transfections were performed in triplicate using SuperFect (QIAGEN Inc., Valencia, CA) or Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) according to the protocol provided by the manufacturer. Briefly: serum-free media (0.1 ml) was aliquoted and DNA added (1 µg of the reporter gene plasmid and 0.025–0.5 µg of the expression vector depending upon whether ER or PR assays were being performed), after vortexing, 10 µl of SuperFect reagent was added and vortexed for 10 sec. Following incubation at room temperature for 5–10 min, 0.6 ml of supplemented DMEM-HG was

added to each sample. The medium containing the transfection complexes was added to the cell monolayer, which had previously been rinsed with PBS. The plates were incubated for 3 h at 37 C in 5% CO₂. After incubation, the plates containing the transfection complexes were rinsed with PBS and 3 ml of supplemented DMEM-HG was added to each well. Lipofectamine transfections were performed as previously described (18). Twenty-four hours later, the medium was replaced with medium containing the compounds of interest at various concentrations (10⁻¹²–10⁻⁶ M). Dimethyl sulfoxide or ethanol was used as vehicle. CAT activity using 5 µg of protein, 10 µg of butyryl coenzyme-A (Sigma MO), 2 × 10⁵ cpm of xylene-extracted [³H]chloramphenicol in 0.25 M Tris-HCl, pH 8.0, was assayed as previously described (19–21). For the luciferase assays, 48 h after transfection the cells were rinsed 1× with PBS without Ca²⁺ or Mg²⁺. After aspiration 600 µl of 1× Passive Lysis Buffer (Promega Corp., Madison, WI) was added to each well of a six-well plate. The plates were incubated at room temperature with rocking/shaking until cells lysed. The cell lysates were transferred to a 1.5-ml microcentrifuge tube and centrifuged for 2 min at 4 C to form clear lysates. Aliquots of cell lysates (20 µl) were transferred to 12 × 75 mm polystyrene tubes (Sarstedt, Newton NC) suitable for use with a luminometer. Reagents used were appropriate for the dual-luciferase assay (Promega Corp., Madison, WI). Samples were read on a Monolight 3010 luminometer (PharMingen, San Diego, CA). Statistical significance was determined using two tailed *t* test.

Receptor binding studies

The relative receptor binding affinities were determined as described by Smith and Kreutner (22). Briefly: an adenovirus-mediated DNA transfer procedure (22, 23) was used to transfect COS-1 cells with 3 µg of ER expression vector (pCMV$_5$-hERα or pCMV$_5$-hERβ). Twenty-four hours later, cells were harvested and whole cell extracts were prepared in TESH (10 mM Tris, pH 7.7, containing 1 mM EDTA, 0.1% monothioglycerol and 0.4 M NaCl). Cell extracts were incubated with 1 pmol [³H]E2 and increasing concentrations (0.005–5000 pmol) of either E2 or the synthetic test compounds for 3 h on ice. Free steroid was separated from receptor-bound steroid by adsorption to hydroxyapatite Bio-Gel HTP gel (Bio-Rad Laboratories, Inc., Hercules, CA). The amount of ER-bound [³H]E2 was quantified by scintillation counting.

Results

Molecular structures.

The structures of the compounds used in this study for analysis are shown in Fig. 1. All compounds are 19-nor T derivatives. The 5α-dihydro and 3α,5α- and 3β,5α-tetrahydro derivatives of NET and GSD, respectively were prepared by chemical double bond hydrogenation of the A-ring as described in *Materials and Methods*.

Effects of A-ring reduction upon NET and GSD transactivation through PR

Figure 2 shows the effects on PR transcriptional activity of NET and GSD and their corresponding A-ring reduced derivatives in HeLa cells transfected with PR$_A$ (Fig. 2, A and B) or PR$_B$ (Fig. 2, C and D) expression vectors. Cells were transfected with an expression plasmid for either PR$_A$ or PR$_B$ with a progesterone (P$_4$)-responsive reporter gene and treated with a 10⁻⁸ M concentration of each steroid, including P$_4$. As shown in Fig. 2, both NET and GSD behaved as full PR agonists. GSD is a more potent transcriptional activator of both PR$_A$ and PR$_B$ than either NET or P$_4$ (Table 1). As expected by the previously reported relative binding affinities to PR (24), double bond hydrogenation resulted in a significant reduction in the transcriptional potency of NET and

FIG. 1. Molecular structures of NET, GSD, and their corresponding A-ring reduced metabolites.
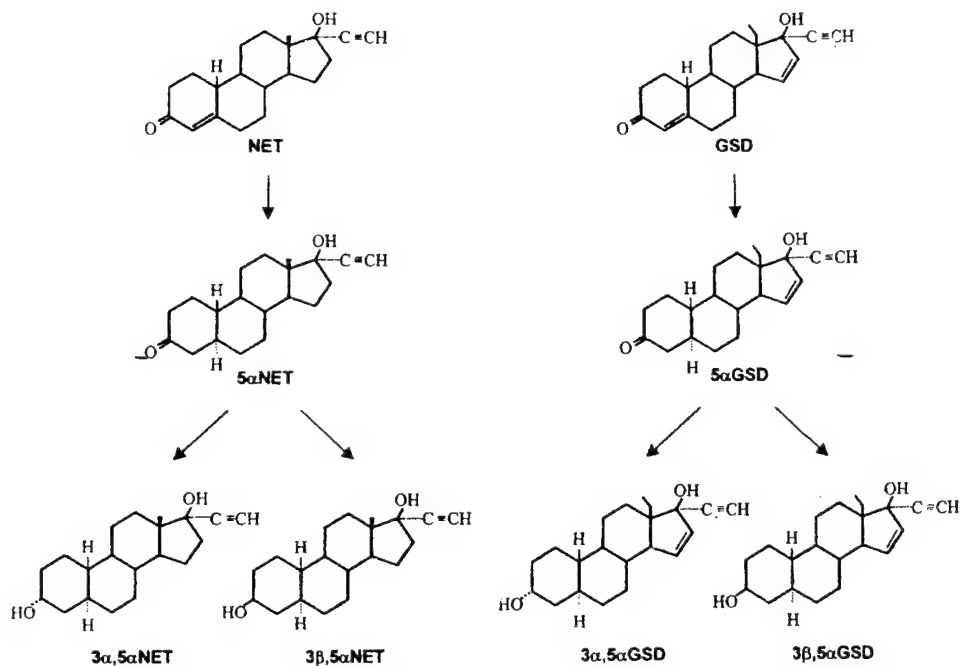
FIG. 2. Activation of PR$_A$ (A and B) and PR$_B$ (C and D) by NET, GSD and their reduced metabolites. HeLa cells that were transiently transfected with the corresponding PR expression vector and the PRE-E1b-CAT reporter were cultured either in the absence (V) or presence of 10^{-8} M of P$_4$ or NET, GSD, or their corresponding synthetic dihydro- and tetrahydro-derivatives. After 24 h, cells were harvested and triplicate dishes were assayed for CAT activity as described in *Materials and Methods* section. Values are the mean ± SD of a representative experiment performed in triplicate. The data are normalized to activity with P$_4$, which is set at 100%. The activity of PRB relative to PRA was approximately 5-fold greater.
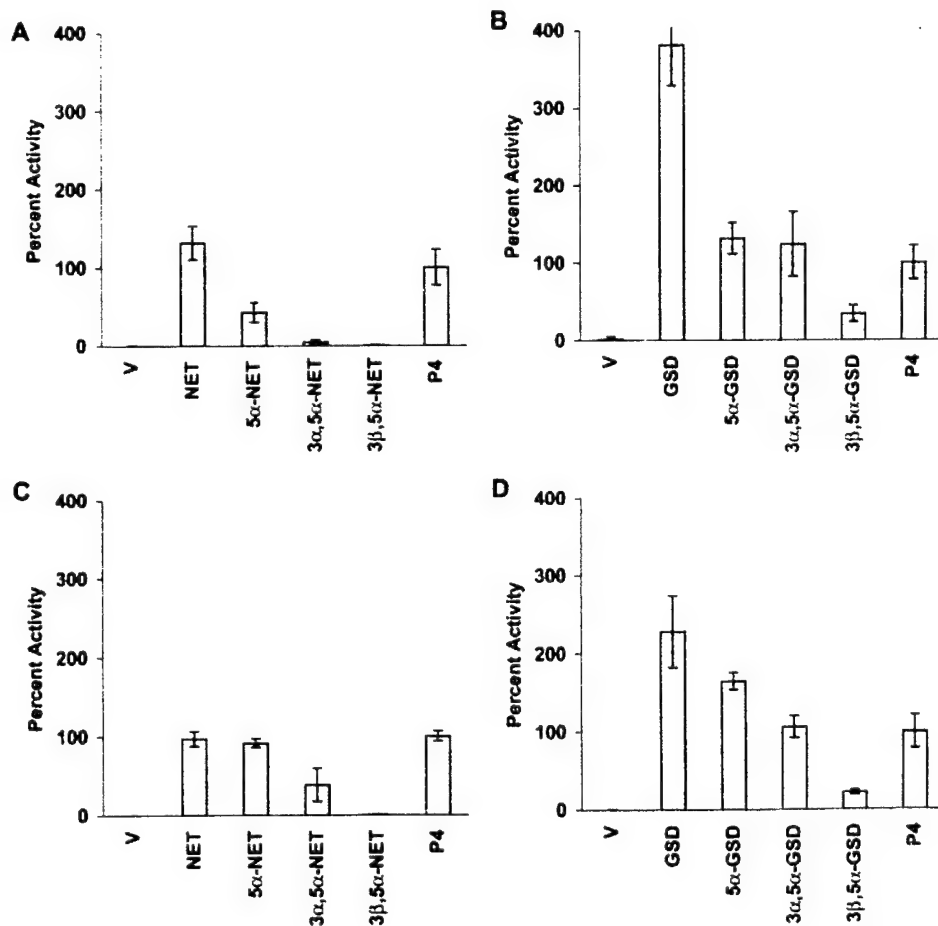
GSD. The 3α,5α- and 3β,5α-tetrahydro derivatives of NET and GSD gave the lowest transactivation through both PRs compared with the other compounds. The 3α,5α- and 3β,5α-

tetrahydro derivatives of NET and GSD have approximately 500- to 1,000-fold difference in transcriptional potency, as judged by their respective EC$_{50}$ values (Table 1), obtained in

dose-response experiments (data not shown), when compared with that obtained with their corresponding nonreduced parent compounds.

Selective transactivation of ER subtypes

Given the well documented *in vivo* and *in vitro* estrogen-like effects of nonphenolic A-ring-reduced derivatives of NET and GSD (9, 13, 25), the synthesized A-ring-reduced metabolites were examined for their separate activities through ERα and ERβ. In these studies, the effects of GSD, NET, and their corresponding dihydro and tetrahydro derivatives on receptor-mediated reporter gene transcription were compared with those of E2. As shown in Fig. 3, out of the eight synthetic steroids tested, both 3β,5α-NET and

$3\beta,5\alpha$-GSD showed the highest induction of ER-mediated reporter gene transcription when HeLa cells were transiently transfected with the ERα and the ERE-E1b-CAT reporter (Fig. 3, A and B). As depicted, these two compounds $3\beta,5\alpha$-NET and $3\beta,5\alpha$-GSD at a concentration of 10^{-8} M exhibited a similar transcriptional activity to that observed with the same dose of E2. Interestingly, a similar concentration (10^{-8} M) of these compounds showed no stimulation of gene transcription through ERβ (Fig. 3, C and D). Similar results were obtained with the 5α-dihydro and the 3α,5α-tetrahydro derivatives of NET and GSD, respectively, but with significantly lower potency for ERα. At all doses used (10^{-12} M to 10^{-6} M), unmodified NET and GSD led to the lowest levels of induction of ER subtype-mediated reporter gene transcription, compared with the derivatized compounds (data not shown).

Figure 4, A and B, shows a comparison of the agonist activity at various doses (10^{-12}–10^{-6} M) of $3\beta,5\alpha$-NET and $3\beta,5\alpha$-GSD on reporter gene transcription through either the ERα or ERβ. As depicted in Fig. 4A, $3\beta,5\alpha$-NET resulted in ERα selective transactivation of reporter gene expression with an EC_{50} value that was 100-fold higher than that obtained with E2. As can also be observed in Fig. 4A, $3\beta,5\alpha$-NET, at the doses of 10^{-12}–10^{-7} M, was unable to stimulate ERβ-mediated reporter gene transcription. Only at the highest concentration tested of this compound (10^{-6} M) was ERβ able to stimulate reporter CAT activity. Similar results were obtained on transcriptional activation through the ERα and

TABLE 1. Stimulatory concentrations (EC_{50}) of natural progesterone and synthetic steroids on transcriptional activation through the progesterone receptor A and B

Steroid	PR_a (mol/liter)	PR_b (mol/liter)
Progesterone	2.90×10^{-8}	8.76×10^{-10}
NET	9.10×10^{-11}	8.16×10^{-11}
GSD	1.36×10^{-11}	1.83×10^{-11}
5αNET	1.26×10^{-9}	1.80×10^{-8}
5αGSD	3.75×10^{-10}	2.29×10^{-9}
3α,5αNET	4.62×10^{-8}	2.56×10^{-8}
3α,5αGSD	1.06×10^{-8}	1.28×10^{-8}
3β,5αNET	6.75×10^{-8}	7.91×10^{-8}
3β,5αGSD	1.01×10^{-8}	3.90×10^{-8}

FIG. 3. Effects of NET, GSD, and their dihydro- and tetrahydro-derivatives on ERα (A and B) and ERβ (C and D)-mediated reporter ERE-E1b-CAT activity. Cells were cultured in the absence (V) or presence of 10^{-8} M of the corresponding natural and synthetic steroids. Values are the mean ± SD of a representative experiment performed in triplicate. The data are normalized to activity with E2, which is set at 100%.
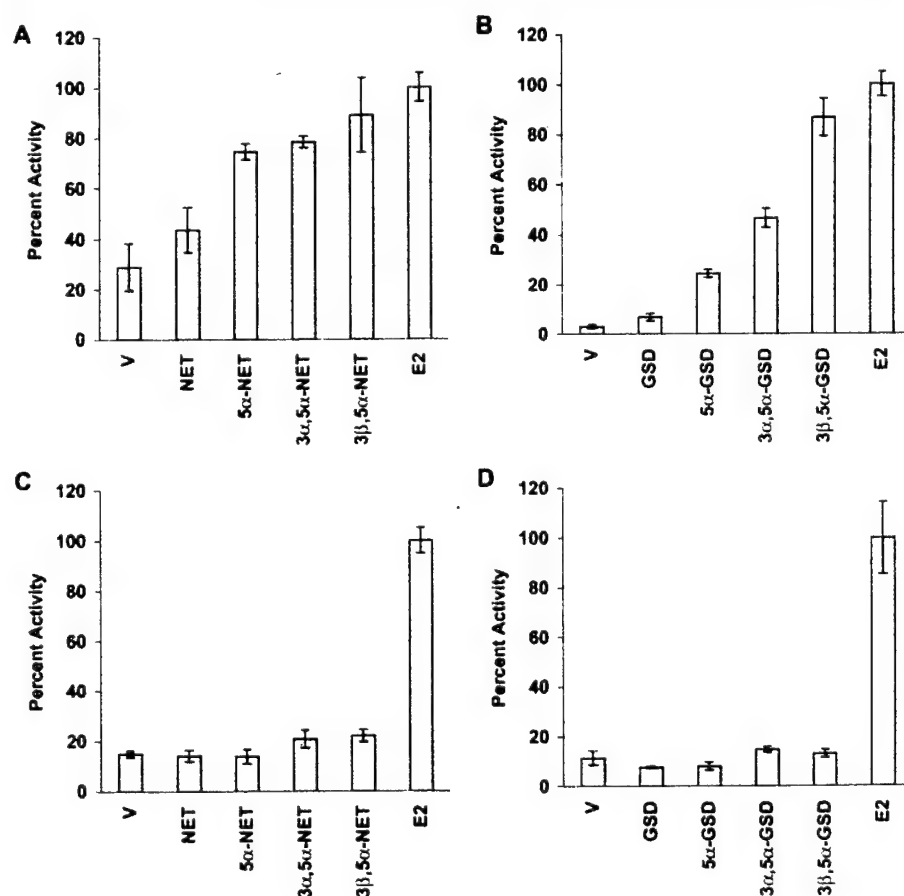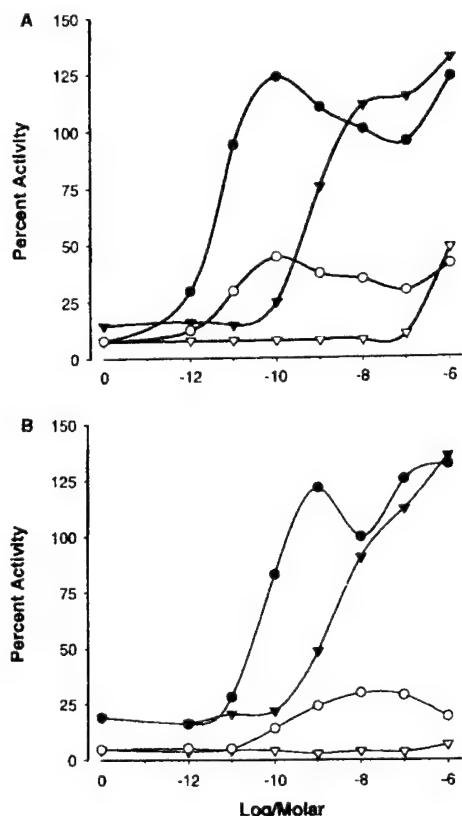
FIG. 4. Dose-dependent activation ERα (*closed symbols*) but not ERβ (*open symbols*) by 3β,5α-NET (A) and 3β,5α-GSD (B). HeLa cells were transiently transfected with expression vectors for ERα (*filled symbol*) or ERβ (*open symbol*) and an ERE-E1b-CAT reporter gene and cultured in the absence or presence of increasing concentrations (10^{-12}–10^{-6} M) of E2 (*circles*) or the 3β,5α derivative (*triangles*) of NET and GSD, respectively. Values are the mean ± SD of triplicate experiments. The data are represented as fold induction relative to the reporter activity plus vehicle alone set as one.

ERβ with 3β,5α-GSD (Fig. 4B). However, 3β,5α-GSD did not activate transcription through ERβ even when used at the highest concentration tested (Fig. 4B). Therefore, 3β,5α-NET and 3β,5α-GSD have increased potency and efficacy for activation of ERα-dependent gene expression relative to ERβ, and these compounds are therefore ERα selective agonists.

Because previously identified ERα-selective ligands were shown to be potent ERβ antagonists, we investigated whether the NET and GSD derivatives act as antagonists, particularly through ERβ (26, 27). HeLa cells were transfected with expression vectors for ERα or ERβ together with the estrogen-responsive reporter gene, and treated with E2 (10^{-9} M) in the presence or absence of increasing concentrations (10^{-8}–10^{-6} M) of NET, or GSD and their corresponding A-ring reduced derivatives, or the antiestrogen 4-hydroxytamoxifen (10^{-7} M). As shown in Fig. 5, with the exception of 4-hydroxytamoxifen, none of the NET (Fig. 5C) or GSD (Fig. 5D) derivatives, at various concentrations (10^{-6} –10^{-8} M), significantly inhibited ERβ-induced reporter gene activation. The increased activities that were observed in Fig. 5D were minor and not reproducible. No inhibition of ERα activity was observed, except with 4-hydroxytamoxifen (Fig. 5, A and B), but this is not unexpected considering the relatively poor

binding affinity of 3β,5α-NET and 3β,5α-GSD in comparison to estradiol, and the ERα agonist activity of these ligands. The antiestrogen 4-hydroxytamoxifen was used to ensure that 3β,5α-NET and 3β,5α-GSD were stimulating target gene expression via ERα. As shown in Fig. 5E, 4-hydroxytamoxifen effectively inhibited the ability of both tetrahydro derivatives to stimulate ERα transcriptional activity.

ER binding studies

The relative binding affinities (RBAs) of the 3β,5α-tetrahydro derivatives of NET and GSD, respectively, were determined in extracts of COS-1 cells transfected with expression vectors for either ERα or ERβ. The numbers represent relative affinities for ERα in comparison to E2, which is set at 100. As shown in Fig. 6A, both compounds showed the ability to displace bound [^{3}H]E2 from the ERα with an RBA of 1.18 and 5.23 for the 3β,5α-NET and 3β,5α-GSD, respectively. In contrast, these compounds behaved as weak competitors for ERβ binding sites (Fig. 6B), as judged by their corresponding RBAs (0.00418 and 0.039 for 3β,5αNET and 3β,5αGSD, respectively).

Promoter and cell specificity of 3β,5α-NET and 3β,5α-GSD activation of ERα

To determine whether 3β,5α-NET and 3β,5α-GSD would activate a natural estrogen responsive promoter selectively through ERα, we transfected the oxytocin promoter luciferase reporter (pROLUC) into HeLa cells with either the ERα or ERβ expression vectors and subsequently treated with the indicated compounds (Fig. 7, A and B). Both 3β,5α-NET and 3β,5α-GSD activated reporter expression from the oxytocin promoter through ERα but not ERβ, whereas the parent compounds NET and GSD had very little effect. Thus, both 3β,5α-NET and 3β,5α-GSD could selectively activate a naturally occurring estrogen responsive promoter. To investigate the cell specificity of the estrogenic effects of 3β,5α-NET and 3β,5α-GSD, we transfected the ERE-E1b-CAT reporter into CHO cells along with an expression vector for ERα (Fig. 7C). Activation of reporter activity by ERα was observed with both 3β,5α-NET and 3β,5α-GSD, but not to the same level as E2; however, they were statistically significant with $P < 0.05$. Both compounds appear to display lower activities in CHO cells than in HeLa cells. This lower activity suggests the relative agonist activity of these compounds may vary depending on cell type. Importantly neither compound stimulated ERβ transactivation in CHO cells.

Discussion

The results of the present study support and extend previous observations from our laboratory that enzyme-mediated hydrogenation of the double bond of 19-nor synthetic-derived T, by formation of A-ring reduced metabolites, determines their biological activity by means of differential interaction with steroid receptors other than PR (9–11, 13, 24). Indeed, the intrinsic estrogenic properties of NET and GSD, two 19-nor T derivatives, most probably lie in their bio-transformation to their corresponding 3β,5α-tetrahydro derivatives (9). In addition, this study clearly shows the
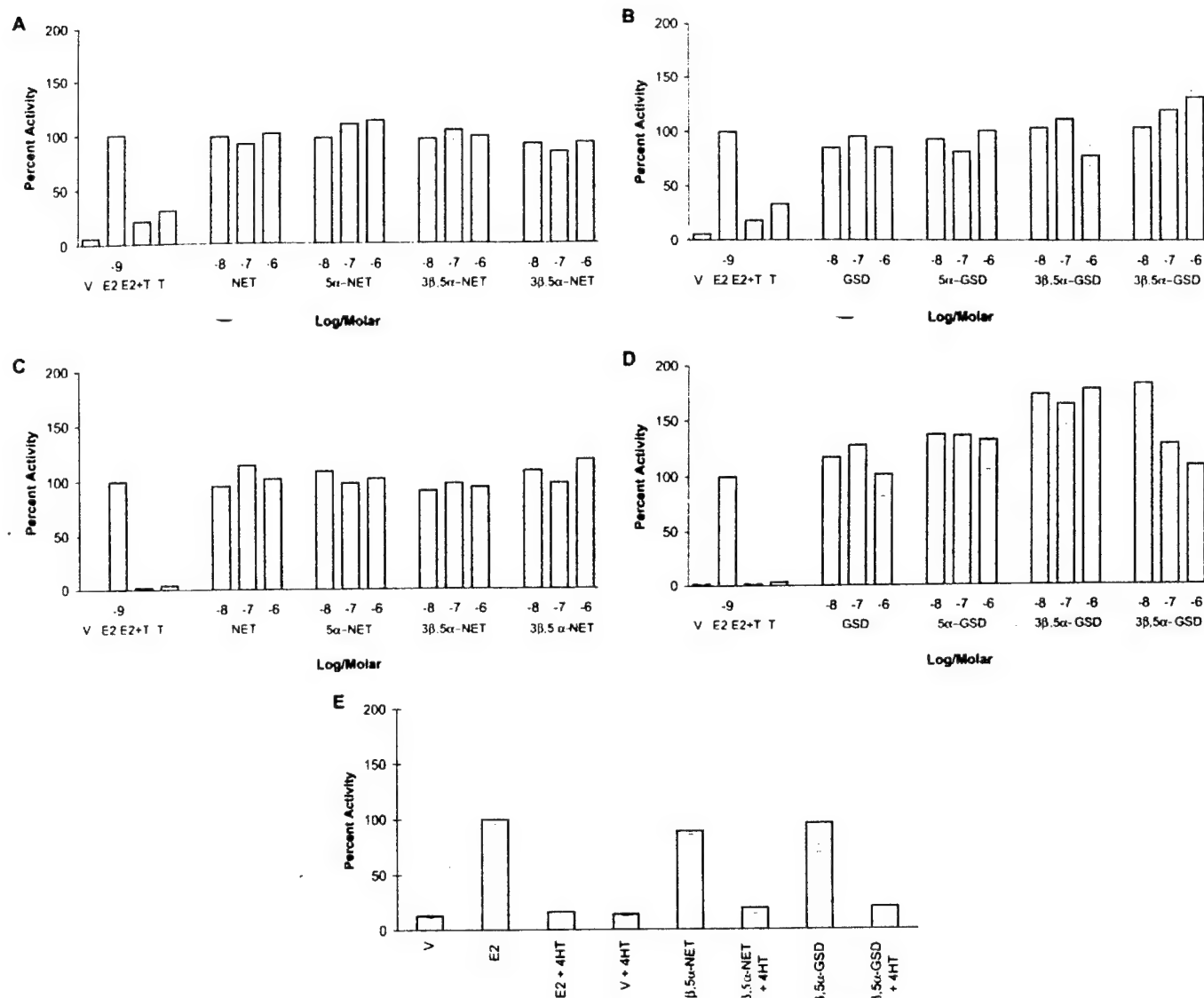
FIG. 5. GSD, NET, and their dihydro and tetrahydro derivatives do not antagonize ERα or ERβ transactivation, but tamoxifen can inhibit their activity. HeLa cells transiently transfected with the expression vector for ERα (A and B) or ERβ (C and D) and an ERE-E1b-CAT reporter gene were incubated with 1 nM E2 in the absence or presence of 10^{-7} M of 4-hydroxytamoxifen (T) or various concentrations (10^{-8} to 10^{-6} M) of either NET (A) or GSD (B) and their corresponding synthetic reduced derivatives. Activation of ERα by 3β,5α-NET and 3β,5α-GSD was also inhibited by the addition of tamoxifen (E). Values are the mean ± SD of triplicate experiments. Control incubations were performed in the presence of only the vehicles (V: dimethyl sulfoxide and ethanol) or (T: ethanol plus T). The data are normalized to activity with E2, which is set at 100%.

specific activation of ERα-dependent reporter gene expression by both 3β,5α-NET and 3β,5α-GSD with little if any activation through other receptors tested. Activation of ERα by 3β,5α-NET and 3β,5α-GSD was observed in both HeLa and CHO cells. In addition, these compounds are capable of activating the oxytocin promoter, a natural estrogen responsive promoter. Therefore, we have demonstrated that these compounds are ERα selective in two different cell lines and on simple and complex natural promoters. This is an important consideration because the relative agonist activity of many ER ligands (*e.g.* selective ER modulators) varies by cell and promoter type. More detailed analyses will be required to gain a broader understanding of the extent of this specificity. Overall, the results are of biological importance in the

sense that although a second ER (ERβ) has been identified, its relevance in estrogen endocrinology is still unclear and compounds such as 3β,5α-NET and 3β,5α-GSD will enable selective *in vivo* studies of only ERα function.

The calculated RBA values from the slopes generated for ERα were very similar to those obtained in previous studies using rat uterus cytosol that involved mainly the ERα (24). This observation, together with previously published data on the ability of NET and 3β,5α-NET to suppress pituitary LH release, including their estrogen-like effects upon the endometrium in castrated female rats (9, 25), supports the selective binding of the 3β,5α-NET to ERα and agrees with the relative distribution of ERα mRNA in different rat and mouse tissues (5, 6, 28, 29). The ERα mRNA is highly ex-
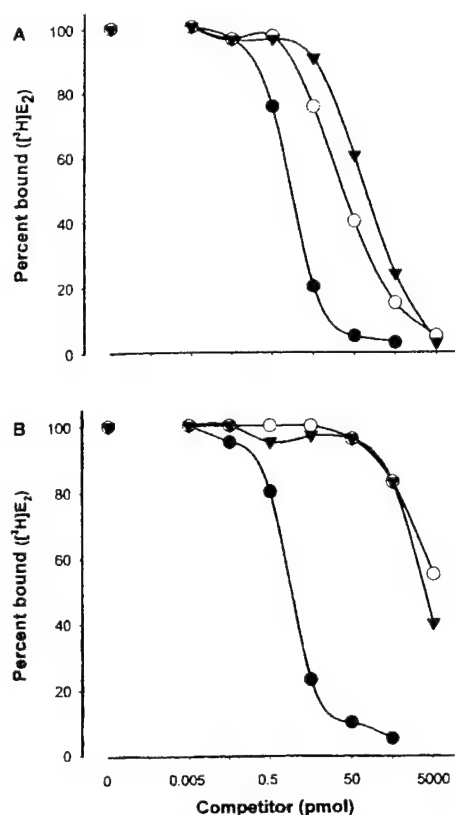
FIG. 6. Relative binding affinity of E2 (*closed circle*), 3β,5α-NET (*open circle*) and 3β,5α-GSD (*triangle*) for ERα (A) and ERβ (B). Extracts from COS-1 cells transfected with expression vectors for either ERα or ERβ were incubated in the presence of 1 pmol [^{3}H]E2 and increasing concentrations (0.005–5000 pmol) of each of the unlabeled competitors. Free from receptor-bound steroid was separated by adsorption to hydroxyapatite. Values are the mean of a representative experiment performed in duplicate.

FIG. 7. Activity of 3β,5α-NET and 3β,5α-GSD on a naturally estrogen responsive oxytocin promoter, and in CHO cells. The pROLUC reporter was transfected into HeLa cells with either ERα (A) or ERβ (B) expression vectors in the presence of E2, NET, GSD, 3β,5α-NET or 3β,5α-GSD. C, The ERE-E1b-CAT reporter was transfected into CHO cells with the ERα expression vector and treated with either E2, 3β,5α-NET or 3β,5α-GSD at 10 μM. Values are the mean ± SEM of representative experiments. The data are normalized to activity with E2, which is set at 100%.

pressed in pituitary, uterus, testis, epididymis and kidney, and 3β,5α-NET estrogenic responses would be expected in these tissues. Furthermore, these compounds may serve as useful tools to discriminate ERα from ERβ functions in tissues (*e.g.* ovary) that express both receptors. Although our findings suggest that 3β,5α-tetrahydro derivatives have receptor-selective actions, a detailed evaluation of the biological effects of these selective ERα agonists in the ERα or ERβ knockout mouse models is, of course, also of relevant interest.

The ability to have preferential ligand selectivity for ERα *vs.* ERβ receptors, as in the case of this study, could help in the process of identifying additional synthetic or naturally occurring steroids with different relative affinities for both ER subtypes. In this regard, it is known that only those C-19 steroids with a hydroxyl group at C-3 and C-17 have significant affinity for both ER subtypes (5). In addition, as shown herein, the relative spatial orientation of the A-ring with respect to the B-ring, as in the case of 5α-reduction and hydroxylation at C-3 of NET and GSD, should also be considered as important structural characteristics for ERα ligand recognition. Whether other alterations in ligand structure, besides those occurring in the A-ring, such as substitutions at C-17 in the α position or the absence of the C-19 methyl

angular (30) in NET and GSD, are important in selective binding to ERα deserves further investigation.

Interestingly, these observations are consistent with previous computer-based quantitative structure-activity relationship studies of ligand-receptor interactions (31). In these studies, comparative molecular field analysis of both ERs revealed that they are sensitive to adding steric bulk at the 17α-position on the steroid ring, suggesting that substitutions in this region will enhance the binding affinity more for ERα rather than ERβ, which is consistent with the experimental findings reported in this study. In addition, the molecular changes elicited by the 19-nor substitution might increase the mobility and electronic density of the A-ring allowing the alignment of the 3β-hydroxy group and the

A-ring hydrogen atoms resembling an estrogen-like environment. There have been several other examples of compounds with differences in the transcriptional activities through both ER subtypes (26, 27). Recently, two novel ligands for ERα and ERβ have been described (26). One, a nonsteroidal triaryl-substituted pyrazole, with a 120-fold agonist potency preference for ERα and the other, a *cis*-diethyl-substituted tetrahydrochrysene prepared as a fluorescent ER ligand, is an agonist on ERα but a complete antagonist for ERβ. In addition, metabolites of methoxychlor were also shown to have similar properties, *i.e.* ERα agonist and ERβ activities (27). Similarly, Kuiper *et al.* (5), reported ERα and ERβ relative binding affinities for a number of estrogen derivatives, as well as nonsteroidal phytoestrogens, such as genistein. In addition, these authors also presented evidence that A-ring reduced natural androgens are more ERβ selective, which may indicate, as described above, the importance of C-17 substitutions and/or the absence of the C-19 methyl angular in ligand-ER interactions. Relative to these previous reports, 3β,5α-NET and 3β,5α-GSD produce better discrimination in relative binding affinity for ERα and ERβ than other compounds examined and this should facilitate studies on the distinct biological roles of both ER subtypes.

Estrogens are known to have important effects on the reproductive system, and targeted disruption of the ERα gene results in sterility in both male and female mice (32). The ERα knockout mice developed normally but were infertile and did not respond to estradiol. These data, together with other observations in the male mice (33), indicate that ERβ alone does not appear to be capable of maintaining normal reproductive function in the ERα knockout mice. Although a complementary role between these two ER subtypes cannot be discarded, it is obvious the importance of ERα in the overall actions of estrogens. In this regard, we have shown (34) the ability of NET to significantly suppress serum LH levels in castrated subjects with testicular feminization syndrome, and the effect of NET and its 3β,5α-tetrahydro reduced metabolite on LH suppression in the long-term ovariectomized female rat (9, 25, 35). Because these conditions are characterized by the absence of androgen action and estrogen-dependent PRs in both testicular feminization syndrome and ovariectomized rats, the LH suppressing activity of NET was probably due to its interaction with hypothalamic-pituitary ERs. Furthermore, administration of 3β,5α-tetrahydro derivatives of NET and GSD is capable of restoring both the content of pituitary PR in the ovariectomized female rat (12, 13) and inducing male sexual behavior when given chronically in combination with 5α-dihydrotestosterone to castrated male rats (36), indicating their intrinsic estrogenic activities. These observations suggest that ERα is involved at the hypothalamic-pituitary unit in terms of gonadotropin regulation as well as in other brain areas controlling sexual behavior. In as much as the clinical dimension of the availability of specific ER subtype ligands has yet to be determined, it is envisioned that identification of compounds with preferential selectivity for ER subtypes would be valuable for the development of new potential therapeutic approaches based on receptor-selective hormonal actions and provide an important tool to examine ERα and ERβ specific cellular and molecular functions.

Overall, the data presented demonstrate that the tetrahydro derivatives of NET and GSD bind and selectively activate gene transcription via ERα. These compounds, in addition to their scientific and therapeutic implications, may also help to identify and differentiate structural features in natural and synthetic ligands responsible for selective binding to ERα and ERβ.

Acknowledgments

References

1. **Tsai M-J O'Malley BW** 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451–486
2. **Tora L, White J, Brou C, et al.** 1989 The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell 59:477–487
3. **Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-Å** 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930
4. **Kuiper GGJM, Shughrue PJ, Merchenthaler I, Gustafsson J-Å** 1998 The estrogen receptor β subtype: a novel mediator of estrogen action in neuroendocrine systems. Front Neuroendocrinol 19:253–286
5. **Kuiper GGJM, Carlson B, Grandien K, et al.** 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138:863–870
6. **Couse JF, Lindzey J, Grandien K, Gustafsson J-Å, Korach KS** 1997 Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. Endocrinology 138:4613–4621
7. **Stillwell WG, Horning EC, Horning MG, Stillwell RN, Zlatkis A** 1972 Characterization of metabolites of steroid contraceptives by gas chromatography and mass spectrometry. J Steroid Biochem 3:699–706
8. **Braselton Jr WE, Lin TJ, Ellegood JO, Mills TM, Mahesh VB** 1979 Accumulation of norethindrone and individual metabolites in human plasma during short- and long-term administration of a contraceptive dosage. Am J Obstet Gynecol 133:154–160
9. **Larrea F, Vilchis F, Chávez B, Pérez AE, Garza-Flores J, Pérez-Palacios G** 1987 The metabolism of 19-nor contraceptive progestins modulates their biological activity at the neuroendocrine level. J Steroid Biochem 27:657–663
10. **Lemus AE, Vilchis F, Damsky R, et al.** 1992 Mechanism of action of levonorgestrel: *in vitro* metabolism and specific interactions with steroid receptors in target organs. J Steroid Biochem Mol Biol 41:881–890
11. **Pérez-Palacios G, Cerbón MA, Pasapera AM, et al.** 1992 Mechanism of hormonal and antihormonal action of contraceptive progestins at the molecular level. J Steroid Biochem Mol Biol 41:479–485
12. **Vilchis F, Chávez B, Pérez AE, García GA, Angeles A, Pérez-Palacios G** 1986 Evidence that a non-aromatizable metabolite of norethisterone induces estrogen-dependent pituitary progestin receptors. J Steroid Biochem 24:525–531
13. **Lemus AE, Zaga V, Santillán R, et al.** 2000 The oestrogenic effects of gestodene, a potent contraceptive progestin, are mediated by its A-ring reduced metabolites. J Endocrinol 165:693–702
14. **Friedman JS, Cofer CL, Anderson CL, et al.** 1989 High expression in mammalian cells without amplification. Bio Technol 7:359–362
15. **Smith CL, Conneely OM, O'Malley BW** 1993 Modulation of the ligand-independent activation of the human estrogen receptor by hormone and antihormone. Proc Natl Acad Sci USA 90:6120–6124
16. **Allgood VE, Oakley RH, Cidlowski JA** 1993 Modulation by vitamin B6 of glucocorticoid receptor-mediated gene expression requires transcription factors in addition to the glucocorticoid receptor. J Biol Chem 268:20870–20876
17. **Burbach JPH, Lopes da Silva S, Cox JJ, Adan RAH, Cooney AJ, Tsai M-J Tsai SY** 1994 Repression of estrogen-dependent stimulation of the oxytocin gene by chicken ovalbumin upstream promoter transcription factor I. J Biol Chem 269:15046–15053
18. **Smith CL, Nawaz Z, O'Malley BW** 1997 Coactivator and corepressor regu-

lation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 11:657–666

19. **Cooney AJ, Tsai SY, O'Malley BW, Tsai M-J** 1992 COUP-TF dimers bind to different GGTCA response elements allowing it to repress hormonal induction of VDR, TR and RAR. Mol Cell Biol 12:4153–4163

20. **Cooney AJ, Leng X, Tsai SY, O'Malley BW, Tsai M-J** 1993 Multiple mechanisms of COUP-TF-dependent repression of VDR, TR and RAR transactivation. J Biol Chem 268:4152–4160

21. **Seed B, Sheen JY** 1988 A simple phase-extraction assay for chloramphenicol acyltransferase activity. Gene 67:271–277

22. **Smith CL, Kreutner W** 1998 *In vitro* glucocorticoid receptor binding and transcriptional activation by topically active glucocorticoids. Drug Res 48:956–960

23. **Allgood VE, Zhang Y, O'Malley BW, Weigel NL** 1997 Analysis of chicken progesterone receptor function and phosphorylation using an adenovirus-mediated procedure for high efficiency DNA transfer. Biochemistry 36:224–232

24. **Chávez BA, Vilchis F, Pérez AE, García GA, Grillasca I, Pérez-Palacios G** 1985 Stereospecificity of the intracellular binding of norethisterone and its A-ring reduced metabolites. J Steroid Biochem 22:121–126

25. **Larrea F, Moctezuma O, Pérez-Palacios G** 1984 Estrogen-like effects of norethisterone on the hypothalamic pituitary unit of ovariectomized rats. J Steroid Biochem 20:841–847

26. **Sun J, Meyers MJ, Fink BE, Rajendran R, Katzenellenbogen JA, Katzenellenbogen BS** 1999 Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor-α or estrogen receptor-β. Endocrinology 140:800–804

27. **Gaido KW, Leonard LS, Maness SC, et al.** 1999 Differential interaction of the methoxychlor metabolite 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane with estrogen receptors a and b. Endocrinology 140:5746–5753

28. **Shughrue PJ, Komm B, Merchenthaler I** 1996 The distribution of estrogen receptor-β mRNA in the rat hypothalamus. Steroids 61:678–681

29. **Shughrue PJ, Lane MV, Merchenthaler I** 1997 Comparative distribution of estrogen receptor-α and β mRNA in the rat central nervous system. J Comp Neurol 388:507–525

30. **Jordan VC, Mittal S, Gosden B, Koch R, Lieberman ME** 1985 Structure-activity relationships of estrogens. Environ Health Perspect 61:97–110

31. **Tong W, Perkins E, Xing L, Welsh WJ, Sheehan DM** 1997 QSAR models for binding of estrogenic compounds to estrogen receptor α and β subtypes. Endocrinology 138:4022–4025

32. **Couse JF, Korach KS** 1999 Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20:358–417

33. **Eddy EM, Washburn TF, Bunch DO, et al.** 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. Endocrinology 137:4796–4805

34. **Pérez-Palacios G, Chavez B, Escobar N, et al.** 1981 Mechanism of action of contraceptive synthetic progestins. J Steroid Biochem 15:125–130

35. **Garza-Flores J, Menjivar M, Cardenas M, Reynoso M, Garcia GA, Pérez-Palacios G** 1991 Further studies on the antigonadotropic mechanism of action of norethisterone. J Steroid Biochem Mol Biol 38:89–93

36. **Morali G, Lemus AE, Oropeza MV, Garcia GA, Pérez-Palacios G** 1990 Induction of male sexual behavior by norethisterone: role of its A-ring reduced metabolites. Pharmacol Biochem Behav 37:477–484

INTRACELLULAR SIGNALING PATHWAYS: NONGENOMIC ACTIONS OF ESTROGENS AND LIGAND-INDEPENDENT ACTIVATION OF ESTROGEN RECEPTORS

Kevin M. Coleman and Carolyn L. Smith

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030

TABLE OF CONTENTS

1. ABSTRACT

Recognition of the complexity of estrogen and estrogen receptor (ER) signaling has substantially increased in the last several years. In their genomic role, estrogens enter the cell and bind to ERs which are members of a superfamily of ligand-regulated transcription factors. However, estrogens also exert non-genomic effects that occur independently of gene transcription. Typically, these relatively rapid events are initiated at the plasma membrane, and result in the activation of intracellular signaling pathways. Regulation of ER transcriptional activity is also complex. Not only do ligands regulate ER-dependent gene expression, but this receptor in the apparent absence of its estrogenic ligand can also be transcriptionally activated by a variety of intracellular signaling pathways. Recent evidence also extends the effects of these signaling pathways to regulating the activity of coactivators, proteins which bind to the ER and amplify its transcriptional activity. Taken together, it is clear that estrogens, ERs and intracellular signaling pathways are intimately linked and this review will explore the relationship between these components of the estrogen-ER signal transduction process.

2. INTRODUCTION

Estrogens are steroid hormones that play critical roles during development and reproduction, which also exert important biological effects in the cardiovascular and skeletal systems and the brain. Specific estrogenic responses have been recognized for decades [e.g. increase in uterine size (1,2)] and the cumulative efforts of many investigators led to the identification (3,4) and cloning of specific nuclear receptors for estrogens (5,6). Thereafter a general scheme of estrogen action emerged in which estrogens diffuse into the cell and bind to intracellular receptors resulting in changes in gene expression and cellular phenotype. However, this mode of estrogen action cannot account for a number of estrogenic responses [e.g. vascular dilation (7)], at least in part, because of the rapidity with which they occur. In another twist to estrogen receptor biology, there are a group of responses that appear to be estrogen-like [e.g. growth factor stimulation of progesterone receptor synthesis (8)] but arise independent of estrogens. Thus, while it is apparent that estrogens and their receptors play important roles in many aspects of physiology, it has become abundantly clear that many diverse mechanisms are utilized to achieve these responses. Many reviews have examined the current understanding of the molecular events through which steroids activate their receptors in their role as transcription factors. It is the purpose of this review to explore the non-traditional mechanisms through which estrogens and the estrogen receptor exert their biological effects.

3. CLASSICAL STEROID SIGNALING

The genomic effects of steroids are mediated by intracellular proteins that belong to the nuclear receptor

superfamily, which includes type I receptors for estrogens (ERα and ERβ), androgens (AR), glucocorticoids (GR), mineralocorticoids (MR), and progestins (PR), and type II receptors for thyroid hormone (TR), vitamin D (VDR), retinoic acid (RAR), and 9-*cis* retinoic acid (RXR). In addition, nearly 30 members of the nuclear receptor superfamily have been identified for which ligands do not exist or have yet to be identified, and these are commonly referred to as orphan receptors (9). In general, steroid (type I) receptors possess six structural domains labeled A through F that are often classified according to their functional properties (10). The A/B region is located in the N-terminus of these receptors and contains the activation function-1 (AF-1) domain. The centrally-located C region encompasses the DNA binding domain and the D region serves as a hinge between the amino- and carboxy-terminal halves of these receptors. The E region serves as the ligand-binding domain (LBD) and encompasses the activation function-2 (AF-2) domain. ERs are notable for their relatively large F domains. These are found at the extreme C-termini of these receptors, and have been shown to play a modulatory role in ERα transcriptional responses (10,11).

Genomic responses to steroids involve the synthesis of mRNAs over the time course of hours to days and are therefore sensitive to inhibitors of transcription such as α-amanitin and actinomycin D. Target gene expression is initiated when hormone enters the cell by diffusion and binds to its cognate receptor (10). In the absence of hormone, type I steroid receptors are complexed to heat shock proteins. Hormone binding induces a conformational change in the receptor, causing it to release heat shock proteins and bind as a homodimer to specific hormone-responsive elements in the promoters of target genes (10). The transcriptional activity of steroid receptors is derived from their ability to recruit coactivators to the promoter of target genes via their AF-1 and AF-2 domains.

Coactivators possess intrinsic enzymatic activities (histone acetyltransferase, ubiquitin ligase, and arginine methyltransferase) and their ability to link steroid receptors with the general transcription machinery is important for activation of receptor-dependent gene expression. To date, more than 40 nuclear receptor coactivators have been identified and the list continues to grow. The first such proteins identified were the steroid receptor coactivator (SRC)/p160 family of coactivators (12-15) which is composed of three members (SRC-1, SRC-2, and SRC-3) that have been referred to by various names (SRC-1/NcoA-1, SRC-2/TIF2/GRIP1/NcoA-2 and SRC-3/RAC3/p/CIP/ACTR/AIB1/TRAM-1). These coactivators possess multiple LXXLL (where L is leucine and X is any amino acid) signature motifs called nuclear receptor (NR) boxes that enable them to interact with the AF-2 domain of ligand-bound receptors. Crystallographic analyses of a GRIP1 NR box peptide bound to the ERα LBD illustrated the significance of these residues in mediating coactivator-receptor interactions (16). The LBDs of ERs are composed of 12 α-helices that in the ligand-occupied receptor are packed tightly together to form a hydrophobic groove on the surface of the receptor. The LXXLL motif and surrounding amino acids of the coactivator NR box form an amphipathic α-helix which make critical contacts with the residues of helices 3, 4, 5 and 12 that constitute the hydrophobic cleft of the LBD. The crystallographic structural data are consistent with earlier nuclear receptor-coactivator interaction models which were based on mutagenesis studies (17-19).

4. NONGENOMIC ESTROGEN SIGNALING

In contrast to these relatively well-characterized mechanisms for genomic steroid action, a growing body of evidence suggests that steroids have cellular effects which are not mediated by the transcriptional activation of their cognate nuclear receptors. According to the classical model of steroid hormone action, these lipophilic molecules must enter the cell to exert their biological effects. The model, however, cannot be reconciled with the increasing evidence which suggests that steroid hormones can also initiate signaling from the exterior of the cell, and may in fact use signal transduction pathways commonly employed by other extracellular signaling molecules such as growth factors. These rapid, steroid-mediated effects are referred to as nongenomic signaling responses and can be distinguished from those mediated by transcription. Nongenomic steroidogenic signaling is usually characterized by several criteria: 1) signaling responses occur within a time course (seconds to minutes) that is generally considered too rapid for transcriptional and translational events to take place; 2) responses are refractory to inhibitors of transcription (*e.g.* actinomycin D) and translation (*e.g.* cycloheximide) and 3) responses can be initiated by steroids conjugated to macromolecules [*e.g.* bovine serum albumin (BSA)] which are theoretically too large to enter cells.

Nongenomic effects have been reported for many classes of steroids (*e.g.* progestins, estrogens, androgens, mineralocorticoids, and glucocorticoids), as well as the secosteroids and thyroid hormones [reviewed in (20,21)]. Each of these classes of ligands exhibits broad and varied nongenomic responses which include modulations in the activity of signal transduction cascades, ion transport and neurotransmitter release (20,21). Moreover, these signaling events have been observed to take place in a number of cell types including those derived from bone, mammary gland, brain and ovary. Importantly, such responses have also been demonstrated in sperm, oocyte and neuronal cells, in which transcription is not thought to play a major role in mediating steroid action. There is considerable overlap in the nongenomic responses observed for the different classes of steroids. This review will focus on the expanding field of nongenomic estrogen signaling action, making occasional comparisons to other steroid hormones.

4.1. Nongenomic effects of estrogens on MAP kinase signaling pathways

To date, approximately 20 members of the mitogen-activated protein kinase (MAPK) family of proteins have been identified in mammals [reviewed in (22)]. These serine/threonine kinases exert their cellular effects by phosphorylating downstream targets such as transcription factors. The MAPKs (*e.g.* Erks, p38, Jnk) are activated through phosphorylation by another family of

43

proteins called MAP/Erk kinases (MEKs; *e.g.* MEK1 and MEK2), which themselves are phosphorylated and activated by MEK kinases (MEKKs; *e.g.* A-Raf, B-Raf, and Raf-1). The Rafs can be activated by various isoforms of ras. In addition, Raf-1 can be phosphorylated by numerous protein kinases, including the Src non-receptor tyrosine kinase, protein kinase C (PKC) and protein kinase B (PKB)/Akt (22). In general, this signaling cascade can be stimulated through extracellular stimuli such as growth factors, which are coupled to intracellular signaling via membrane receptors. Thus, it is clear that MAPKs can be influenced by multiple upstream factors.

Numerous studies have established that estradiol (E2) can rapidly activate extracellular-regulated kinases (Erk1/Erk2) and can influence c-Jun N-terminal kinase (Jnk) activity (23-30). In addition, E2-BSA conjugates also have been able to induce these nongenomic responses (30). The inability of E2-BSA to enter cells was demonstrated by the ability of E2 but not E2-BSA to stimulate ERE-dependent reporter activity (30). Moreover, this experiment indicated that the E2-BSA conjugates did not contain free, unconjugated E2, a potential problem noted to occur with some E2-BSA preparations (31). Collectively, these data suggest that E2 can stimulate MAPKs via a nongenomic mechanism(s).

Recent efforts have been directed toward identifying mechanistic steps involved in the steroidogenic activation of Erk1/Erk2. There is some indication that these mechanisms could involve the classical ERs. Several studies have demonstrated that E2 stimulates Src tyrosine kinase activity and promotes an interaction between ER (ERα or ERβ) and Src kinase both *in vitro* and in cells (23,26,32,33). This interaction takes place between the Src homology-2 (SH2) domain of Src kinase and the LBD of ERα (23,24,26,32,33). Moreover, mutation of tyrosine[537] to phenylalanine, located within the ERα LBD, abolishes the receptor's interaction with Src kinase and thus E2-dependent Src kinase activity. Although this is consistent with a previously established role for SH2 domains in mediating interaction with phosphotyrosine residues (22,33), it should be remembered that mutations in this region are likely to cause pertubations in the structure of the ligand binding domain. Similarly, androgen-induced Erk2 activity is achieved through interactions between a proline-rich region of AR and the SH3 domain of Src kinase (33). The interaction between Src kinase and ERα leads to activation of two Src substrates, Shc and ras GAP (GTPase activating protein)-associated p190, which in turn leads to activation of ras and theoretically activation of MEK kinases (23). Taken together, these results suggest that steroid hormones can stimulate Erk1/Erk2 activity by promoting interactions between classical nuclear receptors and Src tyrosine kinase.

Progestins can also stimulate the Src/Erk signaling pathway, but in contrast to androgens and estrogens which do so through their cognate receptors, progestin-dependent activation of Src/Erk apparently requires both PR and ER (26). Indeed, activation of Erk2 in T47D cells by the synthetic progestin R5020 can be inhibited by either antiprogestin (RU486) or antiestrogen (ICI 182,780) (26). Erk2 activation could not be achieved in R5020-treated T47D-Y cells, a T47D cell subtype that lacks PR (26). Moreover, R5020 could stimulate Erk2 activation in Cos-7 cells only when both PR and ER expression vectors were co-transfected but not when either of the expression vectors for these receptors was transfected alone (26). Although PR and Src kinase could be immunoprecipitated using an antibody to ER, ER but not Src was found in PR immunoprecipitates, which indicated that PR and Src kinase existed in mutually exclusive ER-containing complexes within cells. Therefore since ER, PR, and Src kinase do not appear to form a ternary complex in these cells, the mechanism whereby progestin stimulates Src/Erk signaling is presently unclear (26).

The role of the ER in mediating E2-stimulated Erk1/Erk2 activity in the brain cortex is less clear. It has been shown that Erk2 can be activated in E2-treated cortical tissue samples obtained from either wild type or ERα knockout (ERKO) mice (34). However, a partially ERβ-selective ligand (genistein) had no effect on Erk1 activation in wild type samples and ERβ mRNA expression levels are unchanged in ERKO samples (34). Taken together, these results suggest that neither ERα nor ERβ are required for E2-dependent activation of Erk1. However, this interpretation is complicated by the observation that the antiestrogen ICI 182,780 inhibited E2-dependent Erk2 activity in wild type but not the ERKO tissue cultures (34). Furthermore, an ERα-selective ligand (16α-iodo-17β-estradiol) inhibited basal Erk1 activation in wild type cortical samples (34). Therefore, further experiments are needed to clarify the roles that ERα and ERβ and/or potentially other factor(s) play in mediating E2-dependent Erk activation in the brain cortex.

Another member of the MAPK family, Jnk, can be influenced by treatment of cells with E2 (30). Jnk has been implicated as a factor that can induce apoptosis, in part by causing phosphorylation and thus inactivation of the antiapoptotic factors Bcl-2 and Bcl-xl. In contrast to the stimulatory effect that they exert on Erks, E2 and E2-BSA have been shown to inhibit Jnk activity and thus inhibit apoptosis in human breast cancer cells which have been stimulated with either ultraviolet irradiation or taxol (30). However, E2 treatment did not affect Jnk activity in cells that were not stimulated to undergo apoptosis, suggesting that the E2-stimulated pathway does not affect basal Jnk activity. As expected, the Erk1/Erk2 MAPKs were also activated in response to E2 treatment and are therefore likely to contribute to these E2-mediated anti-apoptotic effects. In contrast to Erks, which were activated within minutes of E2-BSA stimulation, activation of Jnk was observed following six hours of E2-BSA treatment. Parallel experiments were performed which demonstrate that E2-BSA did not significantly stimulate ERE reporter activity under the same experimental conditions, indicating that E2-BSA inhibition of Jnk likely occurred independently of transcriptional events. Estrogen treatment has also been shown to inhibit Jnk activity induced by RANK ligand in the RAW 264.7 monocytic cell line, but

this also required extended periods (24 hours) of hormone treatment (35).

Similar to Jnk-dependent signaling, p38 MAPK (also known as stress-activated protein kinase-2) induction has been correlated with increased apoptosis. Whereas E2 treatment of either MCF-7 or ZR-75-1 cells inhibits apoptosis by inhibiting Jnk activity (30), it has been reported that E2 treatment of HeLa cells stably-transfected with ERα promotes apoptosis by stimulating p38 MAPK (36). However, a subset of these ERα stable cell lines were resistant to E2-induced apoptosis, and upon further analysis it was revealed by gel mobility shift assays that the ER in these cell lines could not bind to EREs, suggesting that E2-induced apoptosis via p38 MAPK was related to classical genomic ER-mediated transcription.

4.2. Nongenomic Effects of Estrogens on Protein Kinase A

The cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)-dependent signaling pathway can also be rapidly stimulated by E2 (37-40). Treatment with either E2 or conjugated E2-BSA promotes development of dopaminergic neurons, as determined by increased neurite outgrowth and arborization (37). These effects could be blocked by an inhibitor (H89) of PKA and the ability of antiestrogens to inhibit these responses seems to vary depending on cell type (37,38). Moreover, the E2-mediated increase in cAMP can be blocked by calcium chelators and therefore appears to require increases in intracellular calcium (38,39). Thus, the mechanisms by which E2 stimulates PKA may be due to crosstalk from other second messenger signaling pathway(s).

4.3. Nongenomic Effects of Estrogens on Protein Kinase C

Although the effects of estrogens on PKC activity have not been explored extensively, there is evidence to suggest that E2 can stimulate activity of this kinase signaling pathway. For example, in rat chondrocytes E2- and E2-BSA-dependent increases in alkaline phosphatase activity and proteoglycan sulfation have been linked to the ability of this steroid hormone to stimulate PKC activity (41). The increase in PKC activity could not be inhibited by the antiestrogen, ICI 182,780 (41). However, a G-protein inhibitor as well as a phosphatidylinositol-specific phospholipase C (PLC) inhibitor (U73122) attenuated PKC activity, which therefore appears to be related to a G-protein-dependent increase in PLC activity. In contrast, E2-dependent stimulation of PKC activity in guinea pig hypothalamic neurons desensitizes G-protein-coupled receptor (GPCR)-mediated events (42), which interestingly, may explain the rapid, negative feedback exerted by estrogens on the hypothalamic-pituitary axis *in vivo* (43). The mechanism by which E2 stimulated PKC in this latter case has not been identified.

4.4. Nongenomic Effects of Estrogens on Calcium

Calcium (Ca^{+2}) is a ubiquitous second-messenger which can influence several intracellular signaling pathways and conversely, these signaling pathways can influence Ca^{2+} levels [reviewed in (44)]. Normally, cytoplasmic Ca^{2+} levels are tightly controlled with relatively high levels found in the extracellular environment and in the endoplasmic reticulum (44). An appropriate stimulus can cause Ca^{2+} to be released from the endoplasmic reticulum and/or be taken up from the extracellular environment, resulting in a rise in cytoplasmic Ca^{2+} (44).

Although E2 stimulation can alter intracellular Ca^{2+}, no generalizations can be made about the mechanism(s) by which estrogens exert this response (28,39,45,46). In many cases these responses do not appear to be blocked by antiestrogens (39,46,47). Some E2-induced changes in cytoplasmic Ca^{2+} can be attenuated either by removing calcium from the extracellular medium or by subjecting cells to L-type Ca^{2+} channel blockers, which indicates that Ca^{2+} influxes through membrane Ca^{2+} channels can make a significant contribution to these cytoplasmic Ca^{2+} changes (39,45,48,49). However, an intracellular calcium chelator demonstrates that estrogen can also mobilize calcium from endoplasmic reticulum stores (28). These hormone effects on membrane calcium channels as well as the endoplasmic reticulum can be mediated through various signal transduction cascades which can involve receptor-tyrosine kinases and G-protein-coupled receptor stimulation of phospholipase C (44). For example, it has been demonstrated that PLC inhibitors (neomycin and U-73122) can attentuate E2-induced changes in Ca^{2+} (49), although in another study a PLC inhibitor had no effect, which might reflect cell type-specific differences in these mechanisms (48). However, the dynamics of Ca^{2+} signaling must also be considered when comparing these responses. Indeed, while it has been demonstrated that PLC inhibitors can block rapid (< 1 min.) increases in Ca^{2+} release, a second, presumably PLC-independent, wave (>5 min.) of Ca^{2+} release was also observed (49). Lastly, PKA and PKC are also thought to play roles in these E2-dependent Ca^{2+} responses (39,46). Thus, multiple signaling pathways can influence E2-dependent changes in cytosolic calcium and this likely depends on cellular context as well as other factors.

4.5. Nongenomic Effects of Estrogens on Endothelial Nitric Oxide Synthase (eNOS)

Endothelial nitric oxide synthase (eNOS) is an intracelluar enzyme that converts L-arginine to L-citrulline and nitric oxide, a by-product which exerts vasodilatory as well as cardioprotective effects. Consequently, the induction of eNOS activity is typically determined by measuring the conversion of radiolabeled L-arginine to radioactive L-citrulline. A role for nongenomic responses is supported by the observation that E2 rapidly stimulates eNOS activity (50-55). Activation of eNOS by E2 was typically inhibited by ICI 182,780. Moreover, in the absence of transfected ER, E2 was unable to stimulate eNOS activity in Cos-7 and CHO cells (52,54), suggesting that E2 induction of eNOS is mediated through classical estrogen receptors.

The ability of E2 to stimulate eNOS was recently associated with ERα-mediated activation of the

45

phosphatidylinositol-3-OH (PI-3) kinase-Akt signaling pathway (53,54,56). Antiestrogens can block E2-mediated activation of PI-3 kinase (56) and this is consistent with the sensitivity of eNOS activation to these antihormones. Activation of PI-3 kinase results in an increase in intracellular. membrane-bound phosphoinositides. Akt via its N-terminal pleckstrin homology (PH) domain is recruited to these membrane-associated phospholipids and becomes phosphorylated by two additional kinases. The activated Akt is subsequently released from the membrane and exerts its cellular effects by phosphorylating downstream targets. It has been shown that ERα interacts directly with PI-3 kinase in a ligand-dependent manner both *in vivo* and *in vitro* via the p85α subunit of this kinase (56). This interaction is important for eNOS activation since E2 is unable to stimulate eNOS activity in p85-null fibroblasts (56). Moreover, an exogenously introduced dominant-negative Akt abolished E2 stimulation of eNOS activity (53). Thus, ERα via the PI-3 kinase/Akt signaling pathway can activate eNOS.

Interestingly, another step in the ERα-eNOS signaling pathway may involve the ability of ERα to be localized in caveolae (50,52). Caveolae are membrane invaginations, that are coated on the intracellullar surface with multiple proteins, the most important being caveolin-1 [reviewed in (57)]. Indeed, in the absence of caveolin-1 these invaginations do not form (57). Caveolin-1 possesses a 20 amino acid sequence called the caveolin scaffolding domain, which facilitates recruitment of several signaling molecules including ERα and PI-3 kinase (57). Thus these caveolae might function as a scaffold that can bring ERα and PI3-kinase together.

4.6. Putative Membrane Binding Proteins for Estrogens

One of the major controversies about the nongenomic effects of steroids has been the nature of the membrane steroid binding protein(s). Several mechanisms could possibly account for the nongenomic effects of steroid hormones. These include: 1) classical nuclear receptors located at the membrane; 2) estrogen binding proteins unrelated to the classical nuclear receptor; and 3) nonspecific steroid effects on the lipid bilayer.

There is some evidence to suggest that E2 binding to cells is mediated by the classical ERs (58-64). *In vitro* binding studies have demonstrated that E2 binds to the membrane of CHO cells transiently transfected with either ERα or ERβ expression vectors (59). These studies were conducted by isolating the nuclear and membrane fractions by centrifugation and subsequently incubating them with radiolabeled E2 and then a crosslinking reagent (59). Following SDS-PAGE and autoradiography of the E2-bound proteins, membrane estrogen receptors were detected although they were much less abundant than nuclear receptors (59). Although this methodology has a high potential for cross-contamination between the two fractions that may skew the relative amounts of membrane to nuclear ER, other approaches also suggest that classical nuclear receptors may be located on the plasma membrane. For example, microscopic analyses are commonly

employed to visualize immunolabeled membranes of whole cells (58,59,62). In non-permeabilized GH₃/B6/F10 rat pituitary cells, membrane ERα could be detected by multiple enzymatically-labeled antibodies suggesting that all receptor domains were exposed to the extracellular environment (62). This raises interesting, unresolved, questions about the nature and topology of the putative membrane-bound ER relative to the plasma membrane. In addition, it is interesting to note that these ERα antibodies can also stimulate release of prolactin from these cells suggesting a link between these estrogen binding sites and a biological response (64).

It has also been proposed that protein(s) unrelated to the classical ER could mediate binding of E2 to the membrane. For example, in mouse macrophages where E2-induced Ca^{2+} fluctuations were not inhibited by antiestrogens, membrane binding sites could be labeled with fluorescently-tagged E2 (E2-BSA-FITC) but not with ER antibodies, despite labeling of intracellular ERα; ERβ was not detected in these cells (65). Surprisingly, confocal laser scanning microscopic analysis revealed that the E2-BSA-FITC was internalized into the cytoplasm over a short time course, suggesting that the putative E2 binding protein(s) could undergo a dynamic shift from the extracellular surface to the cytoplasm (65). Importantly, these internalized molecules did not colocalize with markers for acidic vesicles as would be the case if internalization occurred through the normal route of macrophagic phagocytosis. Moreover, non-specific molecules such as BSA and BSA-FITC were not internalized. The E2-induced alteration in Ca^{2+} as well as E2-BSA-FITC internalization was blocked by co-treating cells with pertussis toxin, indicating that this E2 binding protein(s) might be associated with G-protein(s). In addition to identifying an E2 binding protein which is unrelated to ER, these observations raise the interesting possibility that E2 entry into these cells could be regulated, as opposed to passive E2 diffusion.

Lastly, the possibility has been raised that nongenomic responses in part could be due to nonspecific steroid effects on the lipid bilayer. Alterations in membrane fluidity can be determined by first introducing a fluorophore into the plasma membrane and subsequently measuring hormone influences on membrane-dependent light scattering (66). However, these E2-induced effects on the plasma membrane have been observed to occur typically in the presence of micromolar concentrations of hormone while physiological estrogen concentrations are in the nanomolar range (66). Furthermore, these nonspecific effects have not been correlated with alterations in intracellular signaling. Moreover, it has been demonstrated that the 17α-estradiol stereoisomer has little to no effect in comparison to 17β-estradiol in mediating nongenomic effects (45,46,65,67), and general membrane effects are therefore not likely to contribute significantly to steroid-induced nongenomic activity.

5. LIGAND-INDEPENDENT ACTIVATION OF ESTROGEN RECEPTORS

The ability of steroids to stimulate various signal transduction pathways is interesting in view of the association between cell signaling, receptor phosphorylation and activation of receptor transcriptional activity. In contrast to the relatively well-characterized steroid-dependent activation of transcription, several nuclear receptors (*e.g.* ER, AR, PR, RXR, RAR, and VDR) can be activated in the apparent absence of their cognate hormone by processes referred to as ligand-independent activation (68). In general, the ability of several intracellular signaling pathways (*e.g.* PKA, PKC, MAPK) to stimulate nuclear receptor-dependent transcription is thought to be due to the ability of these signaling pathways to target the receptors and their associated proteins (*e.g.* coactivators, corepressors, heat shock proteins), at least in part by phosphorylation-based mechanisms (68).

5.1. ER Phosphorylation

Nuclear receptors, including ERs, are phosphoproteins (69-72). To date, five *in vivo* phosphorylation sites (Ser^{104}, Ser^{106}, Ser^{118}, Ser^{167} and Tyr^{537}) have been mapped for human ERα (73-77). ERα is hyperphosphorylated in response to hormone, and while these studies suggest that Ser^{118} is the major estrogen-induced phosphorylation site (73,75,76) another study indicates that Ser^{167} is the predominant site phosphorylated in response to hormone (74). This discrepancy might be attributable to cell type-specific differences (COS-1 cells *vs.* MCF-7 cells) in ER phosphorylation or reflect differences in the techniques used to map these residues (73-76). Tyr^{537} is a basal phosphorylation site, which is not further altered upon stimulation with hormone (77), and the role it plays in receptor-mediated transcription is unclear. It has been suggested that phosphorylation of this residue, which is located in the LBD, contributes to the ability of the receptor to dimerize and bind DNA (78). In contrast, mutation of Tyr^{537} to various other amino acids either has little effect or renders the ERα mutants constitutively active, indicating that this residue need not be phosphorylated to attain maximal ERα transcriptional activity (79,80).

Research is ongoing to determine the mechanistic basis for hormone-induced phosphorylation of ERα, and several possible mechanisms have been examined by *in vitro* methods. Ser^{118} is part of a MAPK consensus sequence and the ability of MAPKs (Erk1/Erk2) to phosphorylate this residue in response to EGF stimulation has been observed (81). Somewhat surprisingly, the selective MAPK inhibitor, PD98059, was unable to block E2-induced receptor phosphorylation, measured as mobility upshift (slower migrating band, presumably due to phosphorylation) of ERα by SDS-PAGE, which suggested that E2-induced phosphorylation occurs independent of MAPK (82). Alternate factors that might contribute to E2-mediated phosphorylation of Ser^{118} were therefore explored. Based on studies demonstrating that cyclin-dependent kinase 7 (cdk7) mediated the ligand-dependent phosphorylation of the N-terminal domain of RARα, the ability of cdk7 to phosphorylate ERα was examined

(83,84). Interestingly, in Cos-1 cells the combination of MAT1 and cdk7, two components of the general transcription factor TFIIH complex, phosphorylated Ser^{118} of ERα (83). Moreover, it was demonstrated that the ERα's AF-2 domain interacted with other subunits of TFIIH, suggesting that ligand induces interaction with the TFIIH complex via the ERα LBD, thereby recruiting cdk7 to phosphorylate the AF-1 domain (83). This is consistent with the ability of the two AF domains to synergize in receptor-mediated transcription (85). The cell cycle regulator cyclin A/cdk2 has been shown to phosphorylate Ser^{104} and Ser^{106} *in vitro* and enhance E2-dependent activity in HeLa cells (86,87). Finally, another proposed E2-induced phosphorylation site (Ser^{167}) has been shown to be phosphorylated by casein kinase II *in vitro* (74). Thus, different signaling pathways can mediate phosphorylation on different residues within the AF-1 domain in response to hormone, and this may also contribute to the cell-specific activity of ERα. It is evident from recent studies that receptor phosphorylation also contributes to ERβ transcriptional activity (88,89).

5.2. Ligand-Independent Activation by Protein Kinase A

The ability of ERα to be phosphorylated in response to hormone is consistent with its ability to also be activated by several intracellular signaling pathways. Interest in these alternate signaling mechanisms began with earlier observations that a protein kinase A (PKA) activator 8-bromo-cAMP (8Br-cAMP) and a protein phosphatase inhibitor (okadaic acid) could stimulate the transcriptional activity of the A form of the chicken progesterone receptor (cPR_A) in the apparent absence of hormone (90,91). The neurotransmitter, dopamine, presumably through activating a PKA-dependent signaling pathway was subsequently found to stimulate both cPR_A- and ER-dependent transactivation (90,91). These results were surprising since the view at that time was that nuclear receptors were activated only in response to their cognate ligands. Like dopamine, cholera toxin (a G-protein activator) in combination with 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor), increases intracellular cAMP and thus activates PKA. These pharmacological agents when used to treat primary uterine cells in culture resulted in an increase in PR mRNA expression (92), a known ER target gene. As indicated by the ability of the antiestrogen, ICI 164,384, to block 8Br-cAMP-induced PR gene expression, this response was ER-dependent (92). Transient transfection studies indicated that cAMP activation of ERα does not require either Ser^{118} phosphorylation or the receptor's A/B domain (93). Indeed, an ERα deletion mutant lacking its entire amino terminus is capable of being phosphorylated to the same extent as the full-length receptor (73). Another study, which examined the ability of cholera toxin and IBMX to stimulate transcription of several synthetic ER target genes in CHO and 3T3 cells, revealed that activation also depends on cell and promoter context (94), which could be related to the ability of PKA-dependent pathways to regulate receptor dimerization and DNA binding (95). In addition, the ability of these cAMP/PKA-mediated responses to be blocked by cycloheximide, indicated that *de novo* synthesis

of other cellular factor(s) might contribute to determining cell- and promoter-dependence (96,97).

The ability of the cAMP-dependent signaling pathway(s) to activate cPR$_A$ in the absence of an accompanying increase in receptor phosphorylation suggested that receptor-associated proteins might themselves be targets of phosphorylation and as a consequence, indirectly influence nuclear receptor-dependent transcription (98). Indeed, it has been demonstrated that 8Br-cAMP enhances phosphorylation of the SRC-1 coactivator on amino acids Thr[1179] and Ser[1185] and that mutating these residues to alanines (T1179A and S1185A) reduced coactivator-dependent enhancement of PR activity both for ligand-dependent and ligand-independent responses (99). These residues are thought to play a part in stabilizing SRC-1's interaction with another coactivator protein, p/CAF (99). Similarly, a PKA-dependent signaling pathway enhances the intrinsic transcriptional activity of another ER coactivator, CBP (100-103). The cell-cycle regulator, cyclin D1, has been demonstrated to enhance ER-dependent transcription, although it can also act as a corepressor of AR-dependent gene expression (104-107). Interestingly, the cAMP-mediated signaling pathway increases cyclin D1 association with ER, suggesting that the receptor's enhanced activity is a result of improved interaction with other coactivators (108). Thus, cAMP-mediated receptor-dependent transcription may depend on protein-protein interactions as well as modulating the intrinsic activity of coactivators.

5.3. Ligand-Independent Activation by MAP Kinases

Evidence that MAPK could stimulate ER transcriptional activity comes from observations that growth factors (*e.g.*, EGF and IGF-1) can stimulate ER reporter gene expression in cultured cells (109-111). Importantly, these responses were blocked by the antiestrogen, ICI 164,384, demonstrating that they were ER-mediated (109-111). In contrast to the AF-2 domain of ERα, the AF-1 domain is considered to have constitutive activity and is strongly dependent on several of the mapped serine phosphorylation sites (73). Moreover, activation of the ERα by growth factors leads to phosphorylation of Ser[118] (81,109). Consequently, mutation of this residue to an alanine resulted in loss of EGF-induced activity (109). This phosphorylation was likely mediated by MAPK since overexpression of constitutively activated ras or MAPK kinase resulted in enhanced AF-1 activity (81,109). Conversely, a dominant negative form of MAPK kinase resulted in decreased activity (109). Taken together, ERα activation by EGF appears to be mediated through MAPK. However, another study suggests that EGF-mediated activation of ERα might also occur through phosphorylation of Ser[167] by pp90[rsk1] (112). The Src/Jnk signaling pathway also stimulates ERα AF-1 transcriptional activity, although this does not appear to require Ser[118] phosphorylation (113). Another report also demonstrates that the p38 and the Jnk MAP kinases stimulate ERα transcriptional activity independent of the known receptor phosphorylation sites (114). Thus, multiple factors mediate growth factor-dependent signaling to ERα.

Coactivators also play an important role in the ability of growth factors to activate ERα (115,116). The SRC/p160 family coactivators, AIB1 and GRIP1, can be phosphorylated by MAPK (Erk2) *in vitro* (115,116). Accordingly, AIB1 fused to the GAL4 DNA binding domain stimulated GAL reporter activity when it was co-transfected with a constitutively active MAPK kinase, MEK1 (116). In addition, the p300 general coactivator could be co-immunoprecipitated along with AIB1 in a MEK1-dependent manner suggesting this signaling enhanced protein-protein interaction (116). Moreover, histone acetyltransferase (HAT) activity was detected in anti-AIB1-immunoprecipitated complexes prepared from cells expressing the active MEK1 (116). Similarly, the GRIP1 coactivator is phosphorylated by MAPK (Erk2) on Ser[736] *in vitro* and its EGF-induced intrinsic transcriptional activity is attenuated by mutating this residue to an alanine (115). Furthermore, the S736A mutant's ability to enhance the transcriptional activity of a GAL4 DNA binding domain-CBP coactivator fusion construct was substantially impaired relative to the wild type CBP counterpart (115). Taken together, these results suggest that MAPK phosphorylation of these p160 coactivator family members serve to enhance their interaction with p300/CBP and possibly other coactivators, and can therefore lead to indirect regulation of receptor-dependent transcription.

Some *in vivo* EGF responses have been attributed to this growth factor's ability to stimulate ER activity (8,110,117). In ERα knockout (ERKO) mice, the expression of PR in response to EGF is abolished (8). Importantly, EGF receptor expression, autophosphorylation and ability to stimulate c-Fos activity in response to EGF stimulation were not altered in ERKO compared to wild type mice, indicating that the EGF signaling pathway is intact (8). Recently, EGF-mediated activation of ERα was also found to be involved in the lordosis behavioral response in rodents (117).

5.4. Ligand-Independent Activation by Protein Kinase C

ERα-dependent transcriptional responses can also be influenced by agents that stimulate the intracellular PKC signaling pathway (118-121). Interestingly, the gonadotropin releasing hormone (GnRH) activation of ER reporter genes can be blocked by GF109203X, a PKC inhibitor (121). The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), which activates PKC, increases phosphorylation of the ERα A/B domain, and this is at least partially attributed to Ser[118] phosphorylation (73,76). However, TPA reduces ERα expression levels (120,122) and receptor binding to its estrogen response element (ERE) *in vitro* (123). The implications of these TPA-mediated effects relative to ER transcriptional activity are unknown. The ERα can be activated by TPA alone or in a synergistic manner with TPA plus E2, but ERα-dependent responses can also be inhibited by TPA in a promoter-dependent and cell type-dependent manner (118,122). In contrast to previously mentioned signaling pathways, the ability of PKC to modulate ERα-dependent transcription has received little

attention and the significance of these effects on ERα activation remains to be determined.

6. PERSPECTIVES

In recent years, there has been a strong interest in characterizing non-genomic signaling by estrogens. Perhaps the biggest surprise has been the number of different intracellular signaling pathways stimulated by estrogens, and the variety of different cell and tissue types that appear to respond to estrogenic stimuli from the extracellular compartment. The progress in defining estrogen-induced intracellular signaling pathways has been exciting and has revealed the broad potential of non-genomic steroid action to exert biological effects. Perhaps less clear at the present time is the mechanism(s) by which the estrogen signaling pathways are initiated at the plasma membrane. There is evidence both for and against the classical ERs being located at the plasma membrane in mediating these responses, and this is likely to remain an actively explored question in the years to come.

While it was initially surprising that the activities of ERs could be regulated by intracellular signaling pathways, the evidence supporting this mode of transcriptional regulation for ERα is increasing and ERβ studies are being actively pursued. At the present time, it is not clear whether ligand-independent ER signaling fulfills a role distinct from ligand-induced ER activity or whether cell signaling events merely augment estrogen-induced responses. Most studies indicate that ligand-independent signaling pathways do not serve any unique purpose, but rather seem to mimic steroid-induced responses. However, resolution of questions regarding the transcriptional targets of the ER induced by ligand-dependent versus ligand-independent signaling pathways awaits further experimentation, perhaps by microarray analyses of gene expression patterns. In view of the non-genomic ability of estrogens to induce the same signaling pathways that can activate the ER in a ligand-independent manner, it is possible that there will be significant overlap in the transcriptional targets of ER induced by ligand-dependent and ligand-independent signaling pathways. Perhaps a larger question at the present time is whether it is possible for estrogens to exert non-genomic and genomic responses in the same cell, and if so, whether these events are independent or influence each other. It seems likely that resolution of this question may have a significant impact on how estrogen action is viewed in a number of tissues, including the cardiovascular and skeletal systems, and may therefore provide novel and interesting pharmacological prospects.

7. ACKNOWLEDGMENTS

8. REFERENCES

1. E. B. Astwood: A six-hour assay for the quantitative determination of estrogen. *Endocrinology* 23, 25-31 (1938)

2. R. B. Holden: Vascular reactions of the uterus of the immature rat. *Endocrinology* 25, 539-596 (1939)

3. D. Toft & J. Gorski: A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proc Natl Acad Sci USA* 55, 1574-1581 (1966)

4. E. V. Jensen, T. Suzuki, T. Kawashima, W. E. Stumpf, P. W. Jungblut & E. R. Desombre: A two-step mechanism for the interaction of estrogen with the rat uterus. *Proc Nalt Acad Sci USA* 59, 632-638 (1968)

5. S. Green, P. Walter, V. Kumar, A. Krust, J. M. Bornert, P. Argos & P. Chambon: Human oestrogen receptor cDNA: sequence, expression and homology to V-erb-A. *Nature* 320, 134-139 (1986)

6. G. G. Kuiper, E. Enmark, M. Pelto-Huikko, S. Nilsson & J. Gustafsson: Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 93, 5925-5930 (1996)

7. R. H. Karas & M. E. Mendelsohn: Rapid vasomotor effects of estrogen: men are part of the club. *Chest* 114, 1508-1509 (1998)

8. S. W. Curtis, T. F. Washburn, C. Sewall, R. DiAugustine, J. Lindzey, J. F. Couse & K. Korach: Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. *Proc Natl Acad Sci USA* 93, 12626-12630 (1996)

9. V. Giguere: Orphan nuclear receptors: from gene to function. *Endocr Rev* 20, 689-725 (1999)

10. M.-J. Tsai & B. W. O'Malley: Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Ann Rev Biochem* 63, 451-486 (1994)

11. M. M. Montano, V. Muller, A. Trobaugh & B. S. Katzenellenbogen: The carboxy-terminal F domain of the human estrogen receptor: Role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Mol Endocrinol* 9, 814-825 (1995)

12. C. Leo & J. D. Chen: The SRC family of nuclear receptor coactivators. *Gene* 245, 1-11 (2000)

13. N. J. McKenna, R. B. Lanz & B. W. O'Malley: Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* 20, 321-344 (1999)

14. L. Xu, C. K. Glass & M. G. Rosenfeld: Coactivator and corepressor complexes in nuclear receptor function. *Curr Op Genet Dev* 9, 140-147 (1999)

15. D. Robyr, A. P. Wolffe & W. Wahli: Nuclear hormone receptor coregulators in action: diversity for shared tasks. *Mol Endocrinol* 14, 329-347 (2000)

16. A. K. Shiau, D. Barstad, P. M. Loria, L. Cheng, P. J. Kushner, D. A. Agard & G. L. Greene: The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95, 927-937 (1998)

17. W. Feng, R. C. J. Ribeiro, R. L. Wagner, H. Nguyen, J. W. Apriletti, R. J. Fletterick, J. D. Baxter, P. J. Kushner & B. L. West: Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science* 280, 1747-1749 (1998)

18. D. M. Heery, E. Kalkhoven, S. Hoare & M. G. Parker: A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* 387, 733-736 (1997)

19. H. Y. Mak, S. Hoare, P. M. A. Henttu & M. G. Parker: Molecular determinants of the estrogen receptor-coactivator interface. *Mol Cell Biol* 19, 3895-3903 (1999)

20. E. Falkenstein, H. C. Tillmann, M. Christ, M. Feuring & M. Wehling: Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. *Pharmacol Rev* 52, 513-556 (2000)

21. A. Revelli, M. Massobrio & J. Tesarik: Nongenomic actions of steroid hormones in reproductive tissues. *Endocr Rev* 19, 3-17 (1998)

22. G. Pearson, F. Robinson, T. B. Gibson, B.-E. Xu, M. Karandikar, K. Berman & M. H. Cobb: Mitogen-Activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 22, 153-183 (2001)

23. A. Migliaccio, M. DiDomenico, G. Castoria, A. De Falco, P. Bontempo, E. Nola & F. Auricchio: Tyrosine kinase/p21^{ras}/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J* 15, 1292-1300 (1996)

24. S. Kousteni, T. Bellido, L. I. Plotkin, C. A. O'Brien, D. L. Bodenner, L. Han, K. Han, G. B. DiGregorio, J. A. Katzenellenbogen, B. S. Katzenellenbogen, P. K. Roberson, R. S. Weinstein, R. L. Jilka & S. C. Manolagas: Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* 104, 719-730 (2001)

25. M. Singh, J. G. Setalo, X. Guan, M. Warren & C. D. Toran-Allerand: Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. *J Neurosci* 19, 1179-1188 (1999)

26. A. Migliaccio, D. Piccolo, G. Castoria, M. Di Domenico, A. Bilancio, M. Lombardi, W. Gong, M. Beato & F. Auricchio: Activation of the Src/p21^{ras}/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *EMBO* 17, 2008-2018 (1998)

27. G. E. Swaneck, J. L. Nordstrom, R. Kreuzaler, M.-J. Tsai & B. W. O'Malley: Effect of estrogen on gene expression in chicken oviduct: evidence for transcriptional control of ovalbumin gene. *Proc Natl Acad Sci USA* 76, 1049-1053 (1979)

28. T. Improta-Brears, A. R. Whorton, F. Codazzi, J. D. York, T. Meyer & D. P. McDonnell: Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. *Proc Natl Acad Sci USA* 96, 4686-4691 (1999)

29. H. Endoh, H. Sasaki, K. Maruyama, K. Takeyama, I. Waga, T. Shimizu, S. Kato & H. Kawashima: Rapid activation of MAP kinase by estrogen in the bone cell line. *Biochem Biophys Res Commun* 235, 99-102 (1997)

30. M. Razandi, A. Pedram & E. R. Levin: Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. *Mol Endocrinol* 14, 1434-1447 (2000)

31. P. E. Stevis, D. C. Deecher, L. Suhadolnik, L. M. Mallis & D. E. Frail: Differential effects of estradiol and estradiol-BSA conjugates. *Endocrinology* 140, 5455-5458 (1999)

32. A. Migliaccio, M. Pagano & F. Auricchio: Immediate and transient stimulation of protein tyrosine phosphorylation by estradiol in MCF-7 cells. *Oncogene* 8, 2183-2191 (1993)

33. A. Migliaccio, G. Castoria, M. Di Domenico, A. De Falco, A. Bilancio, M. Lombardi, M. V. Barone, D. Ametrano, M. S. Zannini, C. Abbondanza & F. Auricchio: Steroid-induced androgen receptor-oestradiol receptor β-Src complex triggers prostate cancer cell proliferation. *EMBO J* 19, 5406-5417 (2000)

34. M. Singh, J. G. Setalo, X. Guan, D. E. Frail & C. D. Toran-Allerand: Estrogen-induced activation of the mitogen-activated protein kinase cascade in the cerebral cortex of estrogen receptor-alpha knock-out mice. *J Neurosci* 20, 1694-1700 (2000)

35. N. K. Shevde, A. C. Bendixen, K. M. Dienger & Pike J.W.: Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression. *Proc Natl Acad Sci USA* 97, 7829-7834 (2000)

36. C. C. Zhang & D. J. Shapiro: Activation of the p38 mitogen-activated protein kinase pathway by estrogen or by 4-hydroxytamoxifen is coupled to estrogen receptor-induced apoptosis. *J Biol Chem* 275, 479-486 (2000)

37. C. Beyer & M. Karolczak: Estrogenic stimulation of neurite growth in midbrain dopaminergic neurons depends on cAMP/protein kinase A signalling. *J Neurosci* 59, 107-116 (2000)

38. M. Y. Farhat, S. Abi-Younes, B. Dingaan, R. Vargas & P. W. Ramwell: Estradiol increases cyclic adenosine monophosphate in rat pulmonary vascular smooth muscle cells by a nongenomic mechanism. *J Pharmacol Exp Ther* 276, 652-657 (1996)

39. H. J. Han, Y. H. Lee & S. H. Park: Estradiol-17β-BSA stimulates Ca(2+) uptake through nongenomic pathways in primary rabbit kidney proximal tubule cells: involvement of cAMP and PKC. *J Cell Physiol* 183, 37-44 (2000)

40. S. M. Aronica, W. L. Kraus & B. S. Katzenellenbogen: Estrogen action via the cAMP signaling pathway:

Stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc Natl Acad Sci USA* 91, 8517-8521 (1994)

41. V. L. Sylvia, J. Walton, D. Lopez, D. D. Dean, B. D. Boyan & Z. Schwartz: 17β-estradiol-BSA conjugates and 17β-estradiol regulate growth plate chondrocytes by common membrane associated mechanisms involving PKC dependent and independent signal transduction. *J Cell Biochem* 81, 413-429 (2001)

42. M. J. Kelly, A. H. Lagrange, E. J. Wagner & O. K. Ronnekleiv: Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. *Steroids* 64, 64-75 (1999)

43. T. Yamaji, D. J. Dierschke, A. N. Bhattacharya & E. Knobil: The negative feedback control by estradiol and progesterone of LH secretion in the ovariectomized rhesus monkey. *Endocrinology* 90, 771-777 (1972)

44. D. E. Clapham: Calcium signaling. *Cell* 80, 259-268 (1995)

45. P. G. Mermelstein, J. B. Becker & D. J. Surmeier: Estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor. *J Neurosci* 16, 595-604 (1996)

46. C. M. Doolan, S. B. Condliffe & B. J. Harvey: Rapid non-genomic activation of cytosolic cyclic AMP-dependent protein kinase activity and [Ca(2+)](i) by 17β-oestradiol in female rat distal colon. *Brit J Pharmacol* 129, 1375-1386 (2000)

47. W. P. Benten, M. Lieberherr, G. Giese & F. Wunderlich: Estradiol binding to cell surface raises cytosolic free calcium in T cells. *FEBS Lett* 422, 349-353 (1998)

48. J. K. Huang & C. R. Jan: Mechanism of estrogens-induced increases in intracellular Ca(2+) in PC3 human prostate cancer cells. *Prostate* 47, 141-148 (2001)

49. G. Picotto, G. Vazquez & R. Boland: 17beta-oestradiol increases intracellular Ca^{2+} concentration in rat enterocytes. Potential role of phospholipase C-dependent store-operated Ca^{2+} influx. *Biochem J* 339, 71-77 (1999)

50. H. P. Kim, J. Y. Lee, J. K. Jeong, S. W. Bae, H. K. Lee & I. Jo: Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. *Biochem Biophys Res Comm* 263, 257-262 (1999)

51. P. W. Shaul: Rapid activation of endothelial nitric oxide synthase by estrogen. *Steroids* 64, 28-34 (1999)

52. K. L. Chambliss, I. S. Yuhanna, C. Mineo, P. Liu, Z. German, T. S. Sherman, M. E. Mendelsohn, R. G. Anderson & P. W. Shaul: Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. *Circ Res* 87, E44-E52 (2000)

53. M. P. Haynes, D. Sinha, K. S. Russell, M. Collinge, D. Fulton, M. Morales-Ruiz, W. C. Sessa & J. R. Bender: Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. *Circ Res* 87, 677-682 (2000)

54. K. Hisamoto, M. Ohmichi, H. Kurachi, J. Hayakawa, Y. Kanda, Y. Nishio, K. Adachi, K. Tasaka, E. Miyoshi, N. Fujiwara, N. Taniguchi & Y. Murata: Estrogen induces the Akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. *J Biol Chem* 276, 3459-3467 (2001)

55. Z. Chen, I. S. Yuhanna, Z. Galcheva-Gargova, R. H. Karas, M. E. Mendelsohn & P. W. Shaul: Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin Invest* 103, 401-406 (1999)

56. T. Simoncini, A. Hafezi-Moghadam, D. P. Brazil, K. Ley, W. W. Chin & J. K. Liao: Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 407, 538-541 (2000)

57. A. Schlegel & M. P. Lisanti: Caveolae and their coat proteins, the caveolins: from electron microscopic novelty to biological launching pad. *J Cell Physiol* 186, 329-337 (2001)

58. T. C. Pappas, B. Gametchu & C. S. Watson: Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. *FASEB J* 9, 404-410 (1995)

59. M. Razandi, A. Pedram, G. L. Greene & E. R. Levin: Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: Studies of ERα and ERβ expressed in Chinese hamster ovary cells. *Mol Endocrinol* 13, 307-319 (1999)

60. T. A. Milner, B. S. McEwen, S. Hayashi, C. J. Li, L. P. Reagan & S. E. Alves: Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. *J Comp Neurol* 429, 355-371 (2001)

61. K. S. Russell, M. P. Haynes, D. Sinha, E. Clerisme & J. R. Bender: Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc Natl Acad Sci USA* 97, 5930-5935 (2000)

62. A. M. Norfleet, M. L. Thomas, B. Gametchu & C. S. Watson: Estrogen receptor-alpha detected on the plasma membrane of aldehyde-fixed GH3/B6/F10 rat pituitary tumor cells by enzyme-linked immunocytochemistry. *Endocrinology* 140, 3805-3814 (1999)

63. Y. Berthois, N. Pourreau-Schneider, P. Gandilhon, H. Mittre, N. Tubiana & P. M. Martin: Estradiol membrane binding sites on human breast cancer cell lines. Use of a fluorescent estradiol conjugate to demonstrate plasma membrane binding systems. *J Steroid Biochem* 25, 963-972 (1986)

64. A. M. Norfleet, C. H. Clarke, B. Gametchu & C. S. Watson: Antibodies to the estrogen receptor-alpha modulate rapid prolactin release from rat pituitary tumor

cells through plasma membrane estrogen receptors. *FASEB J* 14, 157-165 (2000)

65.   W. P. Benten, C. Stephan, M. Lieberherr & F. Wunderlich: Estradiol signaling via sequestrable surface receptors. *Endocrinology* 142, 1669-1677 (2001)

66.   R. Clarke, H. W. van den Berg & R. F. Murphy: Reduction of the membrane fluidity of human breast cancer cells by tamoxifen and 17 beta-estradiol. *J Natl Cancer Inst* 82, 1702-1705 (1990)

67.   Y. L. Sylvia, T. Hughes, D. D. Dean, B. D. Boyan & Z. Schwartz: 17β-estradiol regulation of protein kinase C activity in chondrocytes is sex-dependent and involves nongenomic mechanisms. *J Cell Physiol* 176, 435-444 (1998)

68.   N. L. Weigel,Y. Zhang: Ligand-independent activation of steroid hormone receptors. *J Mol Med* 76, 469-479 (1998)

69.   N. L. Weigel: Steroid hormone receptors and their regulation by phosphorylation. *Biochem J* 319, 657-667 (1996)

70.   R. R. Denton, N. J. Koszewski & A. C. Notides: Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. *J Biol Chem* 267(11), 7263-7268 (1992)

71.   S. F. Arnold, J. D. Obourn, M. R. Yudt, T. H. Carter & A. C. Notides: *In Vivo* and *In Vitro* phosphorylation of the human estrogen receptor. *J Steroid Biochem Molec Biol* 52(2), 159-171 (1995)

72.   T. F. Washburn, A. Hocutt, D. L. Brautigan & K. S. Korach: Uterine estrogen receptor in vivo: phosphorylation of nuclear specific forms on serine residues. *Mol Endocrinol* 5, 235-242 (1991)

73.   P. LeGoff, M. M. Montano, D. J. Schodin & B. S. Katzenellenbogen: Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. *J Biol Chem* 269(6), 4458-4466 (1994)

74.   S. F. Arnold, J. D. Obourn, H. Jaffe & A. C. Notides: Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor. *Mol Endocrinol* 8, 1208-1214 (1994)

75.   S. Ali, D. Metzger, J. M. Bornert & P. Chambon: Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. *EMBO J* 12(3), 1153-1160 (1993)

76.   P. B. Joel, A. M. Traish & D. A. Lannigan: Estradiol and phorbol ester cause phosphorylation of serine 118 in the human estrogen receptor. *Mol Endocrinol* 9, 1041-1052 (1995)

77.   S. F. Arnold, J. D. Obourn, H. Jaffe & A. C. Notides: Phosphorylation of the human estrogen receptor on tyrosine 537 *in vivo* and by Src family tyrosine kinases *in vitro*. *Mol Endocrinol* 9, 24-33 (1995)

78.   S. F. Arnold, D. P. Vorojeikina & A. C. Notides: Phosphorylation of tyrosine 537 on the human estrogen receptor is required for binding to an estrogen response element. *J Biol Chem* 270, 30205-30212 (1995)

79.   Q. X. Zhang, A. Borg, D. M. Wolf, S. Oesterreich & S. A. Fuqua: An estrogen receptor mutant with strong hormone-independent activity from a metastatic breast cancer. *Cancer Res* 57, 1244-1249 (1997)

80.   K. E. Weis, K. Ekena, J. A. Thomas, G. Lazennec & B. S. Katzenellenbogen: Constitutively active human estrogen receptors-containing amino acid substitutions for tyrosine 537 in the receptor protein. *Mol Endocrinol* 10, 1388-1398 (1996)

81.   S. Kato, H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, S. Masushige, Y. Gotoh, E. Nishida, H. Kawashima, D. Metzger & P. Chambon: Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270, 1491-1494 (1995)

82.   P. B. Joel, A. M. Traish & D. A. Lannigan: Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase. *J Biol Chem* 273, 13317-13323 (1998)

83.   D. Chen, T. Riedl, E. Washbrook, P. E. Pace, R. C. Coombes, J.-M. Egly & S. Ali: Activation of estrogen receptor alpha by S118 phosphorylation involves a ligand-dependent interaction with TFIIH and participation of CDK7. *Mol Cell* 6, 127-137 (2000)

84.   C. Rochetteegly, S. Adam, M. Rossignol, J.-M. Egly & P. Chambon: Stimulation of RAR-alpha activation function AF-1 through binding to the general transcription factor TFIIH and phosphorylation by CDK7. *Cell* 90, 97-107 (1997)

85.   W. L. Kraus, E. M. McInerney & B. S. Katzenellenbogen: Ligand-dependent, transcriptionally productive association of the amino- and carboxyl-terminal regions of a steroid hormone nuclear receptor. *Proc Natl Acad Sci USA* 92, 12314-12318 (1995)

86.   J. M. Trowbridge, I. Rogatsky & M. J. Garabedian: Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex. *Proc Natl Acad Sci USA* 94, 10132-10137 (1997)

87.   I. Rogatsky, J. M. Trowbridge & M. J. Garabedian: Potentiation of human estrogen receptor α transcriptional activatiton through phosphorylation of serines 104 and 106 by the cyclin A-CDK2 complex. *J Biol Chem* 274, 22296-22302 (1999)

88.   G. B. Tremblay, A. Tremblay, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, F. Labrie & V. Giguere: Cloning, chromosomal localization and functional analysis of the murine estrogen receptor β. *Mol Endocrinol* 11, 353-365 (1997)

89.   A. Tremblay, G. B. Tremblay, F. Labrie & V. Giguère: Ligand-independent recruitment of SRC-1 to estrogen receptor β through phosphorylation of activation function AF-1. *Mol Cell* 3, 513-519 (1999)

90. L. A. Denner, N. L. Weigel, B. L. Maxwell, W. T. Schrader & B. W. O'Malley: Regulation of progesterone receptor-mediated transcription by phosphorylation. *Science* 250, 1740-1743 (1990)

91. R. F. Power. S. K. Mani, J. Codina, O. M. Conneely & B. W. O'Malley: Dopaminergic and ligand-independent activation of steroid hormone receptors. *Science* 254, 1636-1639 (1991)

92. S. M. Aronica & B. S. Katzenellenbogen: Progesterone receptor regulation in uterine cells: stimulation by estrogen, cyclic adenosine 3',5'-monophosphate, and insulin-like growth factor I and suppression by antiestrogens and protein kinase inhibitors. *Endocrinology* 128, 2045-2052 (1991)

93. M. El-Tanani & C. D. Green: Two separate mechanisms for ligand-independent activation of the estrogen receptor. *Mol Endocrinol* 11, 928-937 (1997)

94. B. A. Ince, M. M. Montano & B. S. Katzenellenbogen: Activation of transcriptionally inactive human estrogen receptors by cyclic adenosine 3', 5'-monophosphate and ligands including antiestrogens. *Mol Endocrinol* 8, 1397-1406 (1994)

95. D. Chen, P. E. Pace, R. C. Coombes & S. Ali: Phosphorylation of human estrogen receptor alpha by protein kinase A regulates dimerization. *Mol Cell Biol* 19, 1002-1015 (1999)

96. M. K. el-Tanani,C. D. Green: Interaction between estradiol and cAMP in the regulation of specific gene expression. *Mol Cell Endocrinol* 124, 71-77 (1996)

97. H. Cho, S. M. Aronica & B. S. Katzenellenbogen: Regulation of progesterone receptor gene expression in MCF-7 breast cancer cells: A comparison of the effects of cyclic adenosine 3',5'-monophosphate, estradiol, insulin-like growth factor-I, and serum factors. *Endocrinology* 134, 658-664 (1994)

98. W. Bai, B. G. Rowan, V. E. Allgood, B. W. O'Malley & N. L. Weigel: Differential phosphorylation of chicken progesterone receptor in hormone-dependent and ligand-independent activation. *J Biol Chem* 272, 10457-10463 (1997)

99. B. G. Rowan, N. Garrison, N. L. Weigel & B. W. O'Malley: 8-bromo-cyclic AMP induces phosphorylation of two sites in SRC-1 that facilitate ligand-independent activation of the chicken progesterone receptor and are critical for functional cooperation between SRC-1 and CREB binding protein. *Mol Cell Biol* 20, 8720-8730 (2000)

100. R. P. Kwok, J. R. Lundblad, J. C. Chrivia, J. P. Richards, H. P. Bachinger, R. G. Brennan, S. G. Roberts, M. R. Green & R. H. Goodman: Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* 370, 223-226 (1994)

101. J. C. Chrivia, R. P. S. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy & R. H. Goodman: Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* 365, 855-859 (1993)

102. C. L. Smith, S. A. Onate, M.-J. Tsai & B. W. O'Malley: CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. *Proc Natl Acad Sci USA* 93, 8884-8888 (1996)

103. Y. Kamei, L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S.-C. Lin, R. A. Heyman, D. W. Rose, C. K. Glass & M. G. Rosenfeld: A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85, 403-414 (1996)

104. R. M. Zwijsen, R. S. Buckle, E. M.-Hijmans, C. J. Loomans & R. Bernards: Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. *Genes Dev* 12, 3488-3498 (1998)

105. R. M. L. Zwijsen, E. Wientjens, R. Klompmaker, J. van der Sman, R. Bernards & R. J. A. M. Michalides: CDK-independent activation of estrogen receptor by cyclin D1. *Cell* 88, 405-415 (1997)

106. E. Neuman, M. H. Ladha, N. Lin, T. M. Upton, S. J. Miller, J. DiRenzo, R. G. Pestell, P. W. Hinds, S. F. Dowdy, M. Brown & M. E. Ewen: Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. *Mol Cell Biol* 17, 5338-5347 (1997)

107. C. McMahon, T. Suthiphongchai, J. DiRenzo & M. E. Ewen: P/CAF associates with cyclin D1 and potentiates its activation of the estrogen receptor. *Proc Natl Acad Sci USA* 96, 5382-5387 (1999)

108. J. Lamb, M. H. Ladha, C. McMahon, R. L. Sutherland & M. E. Ewen: Regulation of the functional interaction between cyclin D1 and the estrogen receptor. *Mol Cell Biol* 20, 8667-8675 (2000)

109. G. Bunone, P.-A. Briand, R. J. Miksicek & D. Picard: Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J* 15, 2174-2183 (1996)

110. D. M. Ignar-Trowbridge, K. G. Nelson, M. C. Bidwell, S. W. Curtis, T. F. Washburn, J. A. McLachlan & K. S. Korach: Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. *Proc Natl Acad Sci USA* 89, 4658-4662 (1992)

111. Z. Q. Ma, S. Santagati, C. Patrone, G. Pollio, E. Vegeto & A. Maggi: Insulin-like growth factors activate estrogen receptor to control the growth and differentiation of the human neuroblastoma cell line SK-ER3. *Mol Endocrinol* 8, 910-918 (1994)

112. P. B. Joel, J. Smith, T. W. Sturgill, T. L. Fisher, J. Blenis & D. A. Lannigan: pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol Cell Biol* 18, 1978-1984 (1998)

113. W. Feng, P. Webb, P. Nguyen, X. Liu, J. Li, M. Karin & P. J. Kushner: Potentiation of estrogen receptor activation function 1 (AF-1) by Src/JNK through a serine 118-independent pathway. *Mol Endocrinol* 15, 32-45 (2001)

114.   H. Lee, F. Jiang, Q. Wang, S. V. Nicosia, J. Yang, B. Su & W. Bai: MEKK1 Activation of human estrogen receptor α and stimulation of the agonistic activity of 4-hydroxytamoxifen in endometiral and ovarian cancer cells. *Mol Endocrinol* 14,1882-1896 (2000)

115.   G. N. Lopez, C. W. Turck, F. Schaufele, M. R. Stallcup & P. J. Kushner: Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 co-activator activity. *J Biol Chem* 276, 22177-22182 (2001)

116.   J. F. Font de Mora & M. Brown: AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol Cell Biol* 20, 5041-5047 (2000)

117.   E. M. Apostolakis, J. Garai, J. E. Lohmann, J. H. Clark & B. W. O'Malley: Epidermal growth factor activates reproductive behavior independent of ovarian steroids in female rodents. *Mol Endocrinol* 14, 1086-1098 (2000)

118.   D. M. Ignar-Trowbridge, M. Pimentel, M. G. Parker, J. A. McLachlan & K. S. Korach: Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. *Endocrinology* 137, 1735-1744 (1996)

119.   S. Migliaccio, W. C. Wetsel, W. M. Fox, T. F. Washburn & K. S. Korach: Endogenous Protein Kinase-C activation in osteoblast-like cells modulates responsiveness to estrogen and estrogen receptor levels. *Mol Endocrinol* 7, 1133-1143 (1993)

120.   N. Guilbaud, M. F. Pichon, J. C. Faye, F. Bayard & A. Valette: Modulation of estrogen receptors by phorbol diesters in human breast MCF-7 cell line. *Mol Cell Endocrinol* 56, 157-163 (1988)

121.   F. Demay, M. De Monti, C. Tiffoche, C. Vaillant & M. L. Thieulant: Steroid-independent activation of ER by GnRH in gonadotrope pituitary cells. *Endocrinology* 142, 3340-3347 (2001)

122.   H. Cho & B. S. Katzenellenbogen: Synergistic activation of estrogen receptor-mediated transcription by estradiol and protein kinase activators. *Mol Endocrinol* 7, 441-452 (1993)

123.   M. B. Martin, P. Garcia-Morales, A. Stoica, H. B. Solomon, M. Pierce, D. Katz, S. Zhang, M. Danielsen & M. Saceda: Effects of 12-*O*-Tetradecanoylphorbol-13-acetate on estrogen receptor activity in MCF-7 cells. *J Biol Chem* 270, 25244-25251 (1995)

Send correspondence to : Carolyn L. Smith, Ph.D., Department of Molecular & Cellular Biology, One Baylor Plaza, Houston, TX 77030, Tel: 713-798-6235, Fax:713-790-1275, E-mail: carolyns@bcm.tmc.edu

Dear Author

Re: Ms#675

- During the conversion of your manuscript automatic numbering, formatting (Bold, Italics, etc) and Greek letters are lost. Please review the manuscript carefully to identify and correct such items.

MECHANISTIC DIFFERENCES IN THE ACTIVATION OF ERα- AND ERβ-DEPENDENT GENE EXPRESSION BY cAMP SIGNALING PATHWAY(s)

K.M. Coleman and C.L. Smith. Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas

The cellular effects of estrogens are mediated by two estrogen receptors (ERs), ERα and ERβ, which belong to a large superfamily of nuclear hormone receptors. In contrast to its classical hormone-induced activation, it has been demonstrated that ERα, via a cyclic adenosine monophosphate (cAMP) signaling pathway, can be activated in the apparent absence of hormone. To determine whether ERβ could also respond to this signaling pathway, HeLa cells were transiently transfected with expression plasmids for either receptor along with one of various estrogen response element (ERE)-containing synthetic target genes. Subsequent stimulation of cells with 10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (F/I), which together elevate intracellular cAMP, resulted in robust ERα-dependent transcription of ERE-E1b-CAT and ERE-tk-CAT reporters. More importantly, F/I strongly stimulated ERβ-dependent transcription of these reporters. Induction by F/I was blocked by a protein kinase A (PKA)-selective inhibitor H89, thus confirming that PKA is a mediator of the cAMP response. Deleting the amino-terminal AF-1 domain did not significantly alter ERβ activation in response to F/I, although it reduced ERα activity, indicating that the amino terminus of ERβ does not contribute to cAMP activation of this receptor. Interestingly, F/I was unable to promote ER-mediated (α or β) transcription of several other synthetic and natural target constructs, suggesting that promoter context is a significant determinant of cAMP activation of ER-dependent transcription. Mutational analyses reveal that a putative AP-1 responsive element in ERE-E1b-CAT contributed to ERα, but was absolutely required for ERβ transcriptional responses induced by F/I. Conversely, the ERE was required for cAMP activation of both receptors. These data reveal mechanistic differences in the F/I-induced activation of ERα and ERβ and suggest that activation by cAMP signaling pathway(s) occurs via synergism of transcription factors binding to neighboring DNA response elements and/or their coactivators.

SKF-82958 is a Subtype-Selective Estrogen Receptor-α (ERα) Agonist that Induces ERα Phosphorylation and Synergistic Functional Interactions between ERα and AP-1

Marian R. Walters[1,2], Martin Dutertre[2] & Carolyn L. Smith[2]

[1]Department of Physiology, Tulane Medical School, New Orleans, LA 70112
[2]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030

Key Words: estrogen receptor-α, dopamine, SKF-82958, Jun, phosphorylation, AP-1

Running Title: SKF-82958 activation of ERα

Address Correspondence to: Carolyn L. Smith, Ph.D.
Department of Molecular & Cellular Biology
One Baylor Plaza
Houston, TX 77030
Tel: (713) 798-6235
Fax: (713) 790-1275
Email: carolyns@bcm.tmc.edu

SUMMARY

The transcriptional activity of estrogen receptors (ERs) can be regulated by ligands, as well as agents such as dopamine which stimulate intracellular signaling pathways able to communicate with these receptors. We examined the ability of SKF-82958 (SKF), a previously characterized full dopamine D1 receptor agonist, to stimulate the transcriptional activity of ERα and ERβ. Treatment of HeLa cells with SKF-82958 stimulated robust ERα-dependent transcription from an ERE-E1b-CAT reporter in the absence of estrogen and this was accompanied by increased receptor phosphorylation. However, induction of ERβ-directed gene expression under the same conditions was negligible. In our cell model, SKF treatment did not elevate cAMP levels nor enhance transcription from a cAMP-response element (CRE)-linked reporter. Control studies revealed that SKF-82958, but not dopamine, competes with 17β-estradiol (E2) for binding to ERα or ERβ with comparable relative binding affinities. Therefore SKF-82958 is an ERα-selective agonist. Transcriptional activation of ERα by SKF was more potent than expected from its relative binding activity, and further examination revealed that this synthetic compound induced expression of an AP-1 target gene in a TPA response element (TRE)-dependent manner. A putative TRE site upstream of the estrogen response element and the receptor's amino terminal domain contributed to, but were not required for, SKF-induced expression of an ERα-dependent reporter gene. Overexpression of the AP-1 protein c-Jun, but not c-Fos, strongly enhanced SKF-induced ERα target gene expression, but only when the TRE was present. These studies provide information on the ability of a ligand which weakly stimulates ERα to yield strong stimulation of ERα-dependent gene expression through crosstalk with other intracellular signaling pathways producing a robust combinatorial response within the cell.

INTRODUCTION

The effects of estrogens are mediated by the products of two separate genes; one for estrogen receptor-α (ERα) and another for ERβ. Both are members of the nuclear receptor superfamily of ligand-activated transcription factors. The mechanisms by which ERs activate target gene expression in response to estrogen signaling have been the subject of intense investigation since their respective cDNA cloning (1, 2). Owing to the relatively recent identification of ERβ, the bulk of our knowledge regarding the genomic effects of estrogens is derived from ERα studies. For instance, upon binding to 17β-estradiol (E2), ERα undergoes a series of biochemical alterations including increased phosphorylation and conformational changes as well as the receptor's homodimerization and binding to its target DNA sequence, the estrogen response element [ERE; refs. (3-5)]. ERβ also undergoes conformational changes in response to ligand binding (6, 7), and is phosphorylated *in vivo* (8). With respect to DNA binding, ERβ binds to the same consensus ERE that ERα does, although the latter receptor has an ~4-fold higher affinity for this DNA sequence in comparison to ERβ (9, 10).

While many aspects of the regulation of ERα and ERβ transcriptional activity are quite similar (*e.g.* both bind to EREs and activate transcription in response to E2 binding), a number of differences between these receptors have been noted. For instance, on ERE-containing reporters, ligands such as 4-hydroxytamoxifen exert partial agonist activity on ERα, but act as pure ERβ antagonists (11). This is likely related to differences in the poorly conserved structure and function of the hormone-independent activation function-1 (AF-1) domain which is located in the amino-termini of these receptors (11-13). The carboxy-terminal AF-2 domain is hormone-dependent, reflecting the ability of agonists to bind to the receptor's ligand binding domain and induce a conformational change that creates a binding site for coactivators such as steroid receptor coactivator-1 (SRC-1) and its related family members (14, 15). Intriguingly, this domain is only ~60% conserved between ERα and ERβ, and small differences in the affinity of these two receptors for ligands such as genistein and 16α-bromo-17β-estradiol have been demonstrated (16, 17). Although, several contexts exist whereby the transcriptional activity of ERα is

derived predominantly from the AF-1 or AF-2 domains, in most cells the two activation functions work together to bring about a synergistic activation of transcription (18-20). In contrast, the amino-terminus of ERβ possesses relatively low transcriptional activity in comparison to ERα, and this region has been shown to repress the activity of ERβ's AF-2 domain (11-13).

Estrogen receptors, in addition to their regulation by ligands, can also be activated by extracellular agents that initiate intracellular signal transduction pathways [reviewed in (21)]. For instance, epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF-1) treatment of cells results in initiation of the mitogen-activated protein kinase (MAPK) signal transduction cascade leading to phosphorylation of the ERα serine[118] phosphorylation site and stimulation of ERα transcriptional activity (22-24). Similarly, activation of MAPKs by either EGF treatment or transfection of a dominant active form of ras, induces ERβ phosphorylation and transcriptional activity (8, 25), and this is accompanied by a phosphorylation-dependent recruitment of the SRC-1 coactivator (26). In addition to growth factors, insulin, heregulin, 3,3'-diindolylmethane and the neurotransmitter dopamine can also stimulate ERα transcriptional activity in the apparent absence of ligand (27-31). The latter was the first agent demonstrated to stimulate ERα transcriptional activity in a ligand-independent manner (31). There is no information on the ability of dopamine to stimulate ERβ transcriptional activity. However, dopaminergic activation is not unique to ERα, since this neurotransmitter also activates the human vitamin D (but not glucocorticoid) and chicken progesterone receptors (31, 32). Furthermore, *in vivo* studies have demonstrated that dopamine receptor agonists administered to the third ventricle of the brain lead to initiation of lordosis behavior, a progesterone receptor (PR)-dependent biological response in rodents (33-35).

Dopamine receptors are members of the G protein-coupled receptor superfamily and five genes encoding the D1-D5 subtypes of dopamine receptor have been identified (36). Studies with subtype-specific synthetic dopamine receptor agonists indicate that it is the D1 and/or D5 dopamine receptors that stimulate steroid receptor transcriptional activity (33, 34, 37), and this is associated with D1 and D5 dopamine receptor stimulation of intracellular cyclic 3'-5' adenosine monophosphate (cAMP) production

(36). The mechanisms by which the dopaminergic cell signaling pathway communicates with ERα are not well defined, but it is presumed that increased ERα phosphorylation contributes to this process. In this regard, it is interesting to note that cAMP signaling pathways stimulate ERα transcriptional activity and phosphorylation (38, 39). The chicken PR is also ligand-independently activated by treatment of cells with dopamine or agents that increase intracellular cAMP levels (31, 40). However, cAMP activation of PR-dependent transcription is not accompanied by increased receptor phosphorylation, but rather by an increase in the phosphorylation of the SRC-1 coactivator with which the receptor interacts to stimulate gene expression (41, 42). Taken together, the data support a model in which dopamine and cAMP signaling pathways stimulate gene expression in a receptor-specific manner.

Alterations in the biology of dopamine and its receptors play an important role in a number of human diseases, such as Parkinson's disease, as well as contribute to the reward seeking behaviors associated with cocaine abuse (43-45). The molecular mechanisms of dopamine and dopamine receptor action have therefore been extensively studied, and these efforts have been aided by the identification of high affinity and potent ligands for dopamine receptors. One such compound, SKF-82958 (SKF), is a full dopamine D1-subtype selective receptor agonist with greater potency than dopamine (46, 47). SKF has also been shown to stimulate the transcriptional activity of ERα in SK-N-SH neuroblastoma and MCF-7 breast cancer cells (27, 37). We therefore used SKF-82958 to determine the ability of dopaminergic signaling pathways to regulate ERβ transcriptional activity. We observed that this D1 receptor-selective agonist stimulated the transcriptional activity of ERα, but had negligible agonist activity for ERβ. We also found that SKF-82958 stimulates phosphorylation of ERα to an extent at least as great as that observed for E2. However, SKF-82958 competed with E2 for binding to the receptor, suggesting that it exerts at least some of its effects on ERα transcriptional activity as an ERα ligand. Stimulation of ERα transactivation was greater than that anticipated from its relative binding affinity (RBA) for ERα, and we therefore examined the ability of SKF-82958 to stimulate intracellular signal transduction pathways. While SKF-82958 did not increase cAMP production, it did stimulate pathways leading to activation of AP-1, a transcription

factor(s) known to functionally interact with many steroid receptors (3), and we therefore examined the contribution of AP-1 to SKF-induced ERα transcriptional activity. These studies provide novel information on the ability of a compound to simultaneously stimulate the activity of two transcription factors and in so doing to produce robust stimulation of gene expression through a combinatorial response within the cell.

EXPERIMENTAL PROCEDURES

Chemicals

17β-Estradiol (E2), tetradecanoylphorbol-13-acetate (TPA) and poly-L-lysine were obtained from Sigma Chemical Company (St. Louis, MO). The antiestrogens, ICI 182,780 (ICI) and 4-hydroxytamoxifen (4HT) were gifts from Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK) and D. Salin-Drouin (Laboratoires Besins Iscovesco, Paris, France), respectively. 8-Bromo-cyclic AMP (8Br-cAMP) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Research Biochemicals International (Natick, MA) as were dopamine and the synthetic D1 receptor agonist, SKF-82958. All other chemicals were reagent grade.

Plasmids

The mammalian expression vectors for wild type human ERα (pCMV$_5$-hERα) and its corresponding phosphorylation mutants (S104A/S106A/S118A, S118A and S167A) have been described previously (39) as have the plasmids for human ERβ [pCMV$_5$-hERβ (48)], mouse ERα-Y541A (49), c-Jun [pRSV-jun (50)], c-Fos [pBK-28 (51)] and the pRSV-Not control vector (52). Experiments with deletion mutants of ERα used constructs encoding wild-type ERα (amino acids 1-595), ERα-N282G (amino acids 1-282), ERα-179C (amino acids 179-595), ERα-3x (amino acids 1-595 with three point mutations – D538A/E542A/D545A) and ERα-179C-3x (amino acids 179-595 with the D538A/E542A/D545A

mutations) expressed from the pRST7 vector (20). Plasmids for the SRC-1e, TIF2, RAC3 and CBP coactivators in the pCR3.1 expression vector have been previously described (53). The estrogen-responsive reporter genes, ERE-E1b-CAT (54) and ERE-E1b-LUC (55), have been used in previous studies and both contain nucleotides −331 to −87 of the vitellogenin A2 promoter linked upstream of the adenovirus E1b TATA box. The p-169αCG-CAT and p-100αCG-CAT reporter genes contain portions of the chorionic gonadotropin gene, with or without a CRE, respectively, upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (56). The coll73-CAT reporter and the coll60-CAT reporters contain portions of the collagenase gene upstream of CAT differing in the inclusion or exclusion of a TRE, respectively (57). An expression vector for β-galactosidase, pCMVβ was obtained from Clontech (Palo Alto, CA).

The mammalian expression vector for Flag-hERα was constructed as follows: the yeast expression vector for human ERα, YEPE2 (58) was digested with *Tth*III, blunted, and subsequently digested with *Kpn*I. The resulting fragment was cloned into the *Bam*HI (blunted) and *Kpn*I sites of pSelect-1 (Promega). The ER cDNA was removed from the resulting vector with *Kpn*I and *Sal*I restriction enzyme digestion, and subcloned into the mammalian expression vector, pJ3Ω (59) to create pJ3-hER^{Val400}. The amino-terminal Flag epitope was created by utilizing a PCR approach. Briefly, a 5' primer (5'-GGGGTCGACCATGGACTACAAGGACGACGATGACAAGATGACCATGACCCTCCAC) encoding a methionine residue linked to the Flag epitope sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and the first six amino acids of human ERα and a 3' primer (5'-GCGCTTGTGTTTCAACATTCTCC) corresponding to nucleotides 1017-1039 were used to amplify a 844 base pair nucleotide fragment of the ERα cDNA using pSVMTwt:ER as template (30). The resulting PCR product was digested with *Sal*I and *Not*I and substituted for the *Sal*I-*Not*I fragment of pJ3-hER^{Val400} to create pJ3-Flag-hERα^{Val400}. To replace valine^{400} with cDNA encoding the wild type amino acid (glycine^{400}), the *Not*I-*Sac*I fragment of pSVMTwt:ER (corresponding to amino acids 65 to 595) was substituted for the corresponding region of pJ3-Flag-hERα^{Val400} to create pJ3-Flag-hERα.

Reporter genes lacking the putative TPA response element (TRE) were generated from the parent ERE-E1b-CAT plasmid by deletion or site-directed mutagenesis. In the former case, a 195 base pair fragment of ERE-E1b-CAT was removed by digestion with *Nde*I and *Eco0109*I. The resulting vector was blunt ended with Klenow and religated to yield ERE-E1b-CAT(Δ*Nde-Eco*). To remove the putative TRE sequence by site-directed mutagenesis, the *Ssp*I-*Hind*III fragment of ERE-E1b-CAT was subcloned into pALTER-1 (Promega). Using the PCR Site Directed Mutagenesis System (Life Technologies) and a mutagenic primer, the putative TRE sequence, TGACACA, was mutated to GGACTCA following the manufacturer's recommendations. The latter sequence had been demonstrated previously to prevent AP-1 binding (60). Following sequencing to verify appropriate nucleotide substitutions, a *Nde*I-*Eco0109*I fragment was removed from pALTER-1 and substituted for the comparable region of ERE-E1b-CAT to generate ERE-E1b-CAT(mTRE).

Cell Culture, DNA Transfections and *Trans*-Activation Assays

HeLa cells were routinely maintained in Dulbecco's Modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS). DNA transfections were performed by either Lipofectin (Life Technologies, Grand Island, NY) or adenovirus-mediated approaches (61). For *trans*-activation assays, 24 h prior to transfection, 3 x 10^5 HeLa cells were seeded per well of a 6-well multiwell dish in phenol red-free DMEM containing 5% dextran-coated-charcoal stripped serum (sFBS). For Lipofectin transfections, cells were incubated with the indicated DNAs and Lipofectin according to the manufacturer's guidelines. Six hours later, the DNA/Lipofectin mixture was removed and cells were fed with phenol red-free media containing 5% sFBS and the indicated treatments, and 24 h thereafter the cells were harvested.

To prepare reagents for adenovirus-mediated transfections, replication-deficient adenovirus dl312 was propagated and covalently modified with poly-L-lysine by the method of Cristiano *et al.* (62) modified as described previously (61). CsCl-purified fractions of the modified virus were stored at -80 C until use. Adenovirus-DNA complexes were prepared by adding the lysine-modified adenovirus to

plasmid DNA and subsequently incubating with a 200-fold molar excess of poly-L-lysine (M_r 18-20,000). The adenovirus-DNA-lysine complex was then added to the cells at a virus to cell multiplicity of infection of 500:1. After incubation for 2 h, the medium was replaced with phenol-red-free DMEM supplemented with 5% sFBS. Hormones and/or other treatments, as indicated, were added to the cells 4 h later, and the cells were then harvested 24 h thereafter.

Assays of reporter gene expression were performed on cell extracts prepared by lysing cells by rapid freeze-thaw or addition of lysis buffer (Promega). CAT activity was measured by a phase-extraction method utilizing [^{3}H]chloramphenicol (NEN, Boston, MA) and butyryl-coenzyme A (Pharmacia) as substrates (30, 63). Luciferase activity was measured using the Luciferase Assay System (Promega). Duplicate samples were measured in each experiment and data are presented as the average ± SEM of at least three experiments normalized to protein content measured by BioRad protein assay reagent or β-galactosidase.

Relative Binding Affinity Assays

Relative receptor binding affinities were determined *in vivo* as described previously (64). Briefly, the adenovirus-mediated DNA transfer procedure was used to transfect HeLa cells with 0.25 μg/well of the appropriate expression vector (pCMV$_5$-hERα or pCMV$_5$-hERβ). Twenty-four hours thereafter, media was aspirated from wells, and replaced with phenol-red-free DMEM containing 5% sFBS, ~1 pmol [^{3}H]estradiol (NEN, Boston, MA) and increasing concentrations (ranging from 10^{-10} to 10^{-3} M) of either unlabelled E2, SKF-82958 or dopamine. Following 2 h incubation at 37°C, media was aspirated from plates, cells were washed 3 times in cold PBS, and then incubated in 100% ethanol for 15 minutes at room temperature to extract bound steroid. The amount of ER-bound [^{3}H]estradiol in the ethanol extract was quantified with a Beckman LS 6500 scintallation counter and Biodegradable Counting Scintillant (Amersham, Arlington Heights, IL).

Western Blot Analyses

To assess ER expression, cells were transfected as described above, and harvested for Western blot analysis 24 h thereafter. Cell pellets were resuspended in 50 mM Tris buffer (pH 8.0) containing 400 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.2% Sarkosyl, 100 μM sodium vanadate, 10 mM sodium molybdate and 20 mM NaF, incubated on ice for 60 minutes, and centrifuged at 12,000 g for 10 minutes at 4 C. The resulting supernatant was mixed with SDS-PAGE loading buffer, and resolved by 7.5% SDS-PAGE and electrotransferred to nitrocellulose. Filters were incubated sequentially with primary antibodies against ERα (H222) or the Flag epitope (M2; Sigma) and the appropriate horseradish peroxidase (HRP)-conjugated antibody. Immunodetection was performed with enhanced chemiluminescensce (ECL) reagents as recommended by the manufacturer (Amersham, Piscataway, NJ).

^{32}P Labeling and ERα Immunoprecipitation

Cells were transfected with either pJ3-Flag-hERα or pJ3Ω by the adenovirus method. Eight hours thereafter, media was removed, cells were rinsed with phosphate-free DMEM and re-fed with phosphate-free DMEM containing 5% dialyzed sFCS (HyClone, Logan, UT). Radiolabelled inorganic phosphate (83 μCi/ml media) was added, and cells were incubated for 16 hours. Vehicle (ethanol), 1 nM E2 or 25 μM SKF-82958 was added 90 minutes prior to harvesting cells. Cells were lysed in 50 mM Tris (pH 8.0) containing 5 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 400 mM NaCl, 200 μM sodium vanadate, 10 mM sodium molybdate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 μg/ml aprotinin, 3 μg/ml leupeptin, 3 μg/ml pepstatin, 20 mM disodium p-nitrophenylphosphate, 25 mM β-glycerophosphate, 5 mM L-Phe-Ala, 0.15 mM 1,10-phenanthroline for 60 minutes on ice. Lysates were precleared with rabbit anti-rat IgG and protein-A sepharose prior to the sequential addition of 5 μg of H222 antibody, 10 μg rabbit anti-rat IgG and protein A-sepharose. The immunoreactive sepharose complex was washed with 100 mM Tris buffer (pH 9.0) containing 150 mM NaCl, 1% Triton, 1% Tween-20, 20 mM sodium fluoride, 1 mM sodium vanadate and 10 mM sodium molybdate, and eluted

with 1 M acetic acid. Samples were resolved by 7.5% SDS-PAGE and electroblotted to nitrocellulose and subjected to autoradiography at −80C. Protein levels were subsequently assessed by subjecting this same membrane to Western blot analysis using the anti-Flag M2 antibody, followed by a secondary antibody of horseradish peroxidase-conjugated sheep anti-mouse IgG. Signals were revealed with ECL methods following the manufacturer's instructions (Amersham). The ^{32}P signals were quantitated by a Betagen Betascope 603 Blot Analyzer and normalized to immunoprecipitated protein assessed by Western blot analysis and quantitated by scanning laser densitometry (Model 620, BioRad Laboratories).

RESULTS

SKF-82958 activation of ERα-dependent gene transcription

As previously reported (37), the dopamine D1-selective agonist SKF-82958 (±-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrehydro-1H-3-benzazepine; see **Fig. 1A**), like dopamine, can stimulate ERα transcriptional activity, and this is inhibited by the pure ER antagonist ICI 182,780 (**Fig. 1B**). Dose response studies indicated that half-maximal induction of ER-directed gene expression by SKF-82958 occurred at 2 µM (data not shown). In contrast, maximal dopamine induction of ER-directed gene expression occurs at 100-250 µM (23, 30, 31), suggesting that SKF-82958 is a more potent activator of this response. However, the potency (K_m) and maximum efficacy of SKF-82958 induction of cAMP are similar to that for dopamine in rat brain striatum after treatment *in vivo* (46). This discrepancy suggested that there may be mechanistic differences in the ability of SKF-82958 and dopamine to stimulate ERα transcriptional activity.

To investigate this further relative to the mechanisms of SKF-82958 activation of ERα-dependent gene expression, SKF stimulation of cAMP production in HeLa cells was examined by RIA and compared to the ability of SKF to activate ER-dependent gene expression. No correlation was found, as micromolar doses of SKF-82958 failed to significantly elevate cAMP levels (data not shown). To more

closely mimic conditions under which our *trans*-activation assays are performed, the ability of SKF-82958 to stimulate cAMP response element (CRE)-dependent transcription was assessed. The -169αCG-CAT gene is composed of a fragment of the human chorionic gonadotropin gene promoter containing a CRE element, linked upstream of the CAT reporter gene and is activated by cAMP stimulation of the CREB transcription factor (56). The -100αCG-CAT reporter gene which lacks the CRE was used as a negative control. As shown in **Fig. 1C**, CRE-dependent transcription was stimulated by 8Br-cAMP and, more modestly, by dopamine. However, there was no stimulation of CRE-dependent transcription by E2 or SKF-82958. These results suggest that SKF-82958 is not acting through stimulation of a cAMP-dependent dopaminergic signaling in this system. This result led to a consideration of whether this compound activated ER-dependent gene expression through direct binding to ERα. This question is further underscored by the ring structure of this synthetic D1 receptor agonist (**Fig. 1A**) which is reminiscent of the structures of some ER agonists and antagonists (65).

SKF-82958 binds to ERα and ERβ, but preferentially activates ERα

In order to determine whether SKF-82958 could bind to ERs, whole cell competitive hormone binding assays were performed in HeLa cells transfected with expression vectors for either ERα or ERβ. Cells were incubated with [³H]estradiol and increasing amounts of unlabeled E2, SKF-82958 or dopamine. The displacement curves for ERα and ERβ indicate that SKF-82958 can compete weakly with estradiol for binding to both forms of ER but that dopamine is unable to do so (**Fig. 2, A & B**). The average relative binding affinities of SKF-82958 in comparison to E2 (100) for ERα (0.077 ± 0.018; n=4) and ERβ (0.069 ± 0.009; n=3) are similar and are comparable to those measured by other investigators for low affinity ER agonists such as bisphenol A (16). This result suggests that activation of ER-dependent gene expression may arise through SKF-82958 binding to ERs and serving as a weak receptor agonist, and we therefore wanted to determine whether SKF-82958 could activate both subtypes of ER.

HeLa cells were transfected with expression vectors for human ERα or ERβ and the ERE-E1b-Luc reporter gene, which consists of the estrogen response element (ERE) from the vitellogenin A2 promoter

linked to the TATA box sequence of the adenovirus E1b gene and luciferase reporter gene.. SKF-82958 was not able to significantly activate ERβ-dependent gene expression in comparison to the ability of this compound to stimulate ERα transcriptional activity as shown in **Fig. 3A** or in dose response studies (data not shown). SKF therefore appears to be an ERα-preferential agonist. To ensure that SKF-82958 induction of ERα-dependent gene expression was not due to ligand stabilization of ERα expression, Western blot analysis of ERα expression in cells treated with vehicle, E2 and SKF-82958 was performed, and like E2 and dopamine (30, 53), SKF was found to down-regulate the expression of ERα in HeLa cells (**Fig. 3B**). The ability of SRC family and CBP coactivators to enhance SKF-induced ERα transactivation was also examined. Each was able to significantly enhance the transcriptional activity of ERα (**Fig. 3C**), suggesting the SKF-82958 binding to the receptor allows coactivator-ERα functional interactions.

Characterization of Flag-tagged ERα

Activation of human ERα by E2 is accompanied by increased receptor phosphorylation (39, 66). In order to determine if SKF-82958 alters the biochemical properties of ERα, the phosphorylation status of the receptor was assessed in HeLa cells treated with SKF-82958 *versus* E2. First, an expression vector was constructed for Flag-ERα so that distinct antibodies could be used for immunoprecipitation (anti-ERα) and for receptor quantitation by Western blot analysis (anti-Flag). To demonstrate that the M2 antibody against the Flag epitope reacted with only Flag-ERα, cell lysates were prepared from HeLa cells transfected with either pJ3-Flag-hERα or empty parent vector (pJ3Ω), and subjected to Western blot analysis. The M2 antibody detected an appropriately sized band in HeLa cells transfected with pJ3-Flag-hERα, but not in mock-transfected cells (**Fig. 4A**). In a separate experiment, the hERα antibody, H222, was used to ensure that the protein encoded by the pJ3-Flag-ERα expression vector was immunoreactive with ERα antibodies. As expected, Western blot analysis demonstrated that the Flag-ERα migrated with a slightly lower mobility than wild type ERα (**Fig. 4B**).

The transcriptional activity of Flag-ERα was compared to wild type ERα in transient transfection experiments to ensure that the fusion of the Flag epitope to the N-terminus of ERα did not adversely affect the relative ability of the chimeric receptor to activate expression of a synthetic target gene. HeLa cells were transfected with the ERE-E1b-Luc reporter gene, as well as an expression vector for β-galactosidase (pCMVβ), and increasing amounts ($0 \rightarrow 1000$ ng) of expression vectors for wild type or Flag-tagged ERα. In cells treated with 1 nM E2 both receptors exhibited comparable transcriptional activities in the linear portion of the dose response curve (**Fig. 4C**). Only when very high levels (≥ 500 ng) of the expression vectors were introduced into cells was a modest reduction in activity observed for Flag-ERα relative to wild type ERα. Equivalent amounts of vectors for wild type and epitope-tagged ERα were then transfected into HeLa cells and the ability of each receptor to activate transcription following SKF-treatment was determined. Both receptors were activated to an equivalent extent by SKF-82958 (**Fig. 4D**). Taken together these data indicate that the transcriptional activity of Flag-ERα stimulated by either the natural ligand (E2) or the weakly estrogenic SKF-82958 is comparable to untagged ERα, and Flag-ERα was used therefore for analysis in subsequent phosphorylation studies.

SKF-82958 Induces Phosphorylation of Flag-ERα

In order to determine if activation of ERα-dependent gene expression by SKF-82958 is accompanied by alterations in the biochemical properties of the receptor, Flag-ERα was expressed in HeLa cells using the adenovirus transfection method. Cells were subsequently radiolabeled with [^{32}P]orthophosphate, and treated with vehicle, 1 nM E2 or 25μM SKF-82958 for 90 minutes. Flag-ERα was immunopurified with the H222 anti-ERα antibody, resolved by 7.5% SDS-PAGE, and electrotransferred to nitrocellulose. The resulting blot was subjected to autoradiography to visualize the relative amount of phosphate incorporated into receptor, and was subsequently subjected to Western blot analysis with an anti-Flag antibody (M2) to quantitate relative receptor expression levels. A representative blot indicates that SKF-82958 significantly increased the overall phosphorylation level of ERα relative to ^{32}P incorporation observed in

cells treated with vehicle alone (**Fig. 5A**). As expected, the phosphorylation level of Flag-ERα isolated from cells treated with E2 was also significantly increased in comparison to basal levels. When protein levels were taken into account, the data averaged from 4 experiments indicate that estradiol increased ER phosphorylation by 1.7 ± 0.4-fold, while SKF treatment increased ER phosphorylation by 2.2 ± 0.4-fold (**Fig. 5B**).

AF-1 Phosphorylation Sites are not Required for Activation of ERα by SKF-82958

In human ERα, serines 104 and/or 106, 118, and 167 have been identified as phosphorylation sites (39, 66, 67). Serine[118] is the major phosphorylation site induced in estrogen-treated cells (39, 66), and substitution of alanine for this serine reduces E2-dependent and blocks EGF-stimulated ERα transcriptional activity (22, 23, 39, 66). Therefore, to determine if phosphorylation of serine[118] or other known N-terminal phosphorylation sites are critical for activation of the receptor by the SKF signal transduction pathway(s), the ability of this putative ligand to stimulate the activity of ER phosphorylation site mutants was assessed. The ability of E2 to stimulate the transcriptional activity of the S118A and S167A mutants was not significantly different from wild type ERα while the activity of the S104A/S106A/S118A ERα triple mutant was reduced to approximately 75% of the activity of wild type ERα. Although SKF-82958 was able to stimulate the transcriptional activity of each amino-terminal phosphorylation mutant, activation of S118A (2.7 ± 0.3-fold) and S104A/S106A/S118A (2.5 ± 0.5-fold) ERα mutants was decreased relative to the ability of this compound to activate either wild type (4.5 ± 0.3-fold) or the S167A (4.7 ± 0.2-fold) mutant (**Fig. 6A**). The effect of mutating the only known C-terminal phosphorylation site on SKF activation of ER was tested using a mouse tyrosine[541] to phenylalanine[541] mutant (49). Activation of this mutant by SKF and E2 was comparable to wild type ERα activity. This data indicated that several N-terminal phosphorylation sites contribute to, but are not required for, activation of ERα in response to SKF-82958 treatment. In addition, the phosphorylation site mutations

70

had no greater effect on SRC-1, TIF2, RAC3 or CBP coactivation of SKF-induced ERα transcriptional activity than for E2-stimulated responses (data not shown).

Functional Domains of ERα Required for SKF-82958 Activation

To more generally test the regions of ER required for SKF activation, the ability of this compound to stimulate the transcriptional activity of a series of ER mutants (**Fig. 6B**) was tested. Mutation of the AF-2 domain (D538A/E542A/D545A) in the ERα-3x mutant reduced the ability of E2 and SKF to stimulate ER activity by ~64% and ~78%, respectively, suggesting that the C-terminal AF-2 domain contributes to both mechanisms of activation (**Fig. 6C**). An ER mutant lacking the ligand binding and F domains (N282G) was not activated by SKF-82958 or E2 treatment, and this is in agreement with previous studies in SK-N-SH neuroblastoma cells in which the C-terminus of ERα was required for SKF-82958 activation of target gene expression (37). Deletion of the N-terminal AF-1 domain reduced E2-dependent transcriptional activity by ~62%, and SKF-dependent gene expression by ~70% in the ERα-179C mutant in comparison to wild type receptor. While deletion of the A/B domain in conjunction with the 3x mutation yielded an ER mutant (ERα-179C-3x) unable to activate gene expression in comparison to the empty parent vector. Taken together these data suggest that the N- and C-terminal domains of ERα both contribute to receptor activity stimulated by SKF-82958.

SKF-82958 Activates Gene Expression from a TPA response element-containing promoter

A growing body of evidence indicates that most receptors, whether membrane or nuclear, activate and/or interact with numerous signaling pathways. The dual actions of SKF-82958 in activating dopamine D1 receptors and ERα provided an opportunity to explore the impact of multiple signaling mechanisms induced by a multifunctional activator on nuclear receptor-induced transcription. Although SKF-82958 did not appear to appreciably increase cAMP levels in HeLa cells, activation of dopamine D1 receptors has been shown to stimulate the activity of PKC (68, 69). We therefore examined whether SKF treatment of cells could stimulate the activity of a sequence-specific transcription factor, AP-1, which is a

downstream target of the PKC pathway (70). AP-1 is composed of either homo- or heterodimers within the Jun family (c-Jun, JunB and JunD) or between heterodimers of the Jun and Fos (c-Fos, FosB, Fra1 and Fra2) families (71). HeLa cells were transfected with a coll73-CAT reporter, which contains a TPA responsive element (TRE) to which the AP-1 proteins c-Jun and c-Fos bind, or coll60-CAT reporter plasmid lacking the TRE (**Fig. 7A**) and treated with ethanol (vehicle), 1 nM E2, 100 nM TPA, 10 μM SKF-82958 or 100 nM 4HT. TPA strongly induced TRE-dependent gene expression from coll73-CAT, whereas neither E2 nor 4HT resulted in transcriptional activation (**Fig. 7B**). In contrast, treatment with SKF-82958 resulted in weaker, but significant ($p<0.05$), stimulation of TRE-dependent transcriptional activity. None of the treatments increased transcription from a reporter gene (coll60-CAT) lacking the TRE enhancer.

Enhanced SKF-82958 stimulation of ERα-dependent gene transcription by an upstream TRE

Since SKF weakly stimulated TRE-dependent gene expression and the ERE-E1b-CAT reporter gene contains a putative TRE site in the vector backbone ~255 base pairs upstream of the ERE, we examined the contribution of SKF activation of TRE binding factors to SKF induction of ERα-dependent gene expression. Thus, SKF-82958 or E2-induced CAT expression were compared in the intact ERE-E1b-CAT reporter *versus* constructs in which the putative TRE site was eliminated by deletion (Δ*Nde-Eco*) or point (mTRE) mutagenesis (**Fig. 8A**). The latter point mutant was examined to rule out the possibility that the deletion mutant introduced structural perturbations or removed other cryptic DNA sequences from the reporter that might alter transcriptional responses. As shown in **Fig. 8B**, significant CAT expression was induced by treatment with E2 or SKF-82958 from either the intact or mutant forms of the ERE-E1b-CAT reporter gene. Moreover, the fold-induction by E2 was similar for the three reporter genes, whereas SKF-82958 induction of CAT gene expression was diminished by ~23% and ~28%, when the TRE was deleted or mutated, respectively. The similarity in SKF-82958 effect on gene expression between the reporters generated by deletion versus site directed-mutagenesis is consistent with the interpretation that it is the upstream TRE element, rather than some other element or structural alteration,

that contributes to the magnitude of SKF-82958-induced ER transactivation under these conditions. Moreover, in experiments in which a *Cla*I to *Bgl*I linear fragment of the ERE-E1b-Luc plasmid encompassing just the ERE, E1b and luciferase sequences was transfected into HeLa cells with an ER expression plasmid, SKF stimulation of ERα activity relative to E2 was 50% the level seen for unaltered (circular) target gene (data not shown). Taken together, these results support the hypothesis that TRE elements in reporter plasmids may enhance, but are not required for, induction of ER-dependent gene transcription by multifunctional ligands such as SKF-82958.

Since TRE-dependent activity significantly enhanced SKF activation of ER-dependent gene expression, and since c-Jun has been shown to bind to the amino-terminus of ERα (57), we wanted to ensure that the LBD and AF-2 could support SKF activation of gene expression in the absence of AF-1. We therefore examined the ability of ERα-179C to be activated by SKF-82958 in the absence of the upstream TRE. As shown in **Fig. 8C**, loss of the reporter's TRE site and the receptor's AF-1 domain significantly compromises the ability of SKF-82959 to activate ERα-dependent gene expression, consistent with the interpretation that both the AF-1 and TRE contribute to SKF-induced transcriptional activity. Taken together, these data suggest that SKF-82958 on its own is a weak ERα agonist and that the robust activation seen with full-length receptor is a result of the synergistic activation of ER and cellular factors, such as c-Jun or c-Fos, that can bind to the TRE (60).

Effect of AP-1 overexpression on ERα transactivation by SKF-82958

The above observations suggest that transcription factors able to interact with the TRE binding site can contribute to ERα-mediated gene expression stimulated by SKF-82958. Protein-protein interactions have been reported between the AP-1 protein c-Jun and ER, but not between c-Fos and ER, and occur principally through the N-terminal AF-1 domain of the ER protein (57). In order to further investigate the ability of SKF to synergistically activate ER/AP-1-dependent transcription, we tested the hypothesis that increased Jun/Fos expression would enhance SKF-82958 activation of ER-dependent transcription. HeLa

cells were co-transfected with expression plasmids for c-Jun, c-Fos, or equivalent levels of c-Jun+c-Fos (12.5-100 ng/well), with total DNA/well maintained constant by altering the levels of co-transfected empty plasmid. Jun overexpression resulted in strong and significant increases in basal, E2 and SKF-82958 induced transcription from ERE-E1b-CAT but not from reporter genes lacking the TRE (ΔNdeI-Eco), suggesting that c-Jun activated transcription was primarily dependent on the intact reporter's TRE and not through ERα binding (**Fig. 9A**). Fos overexpression resulted in only very modest increases in the effects of E2 and SKF-82958, with no significant effect on basal activity (**Fig. 9B**). The result from the combination of c-Jun with c-Fos was similar to that of c-Jun alone (**Fig. 9C**). In all experiments performed with the ERE-E1b-CAT(ΔNde-Eco) reporter construct lacking the TRE, no significant increases in transcriptional activation were induced by AP-1 overexpression (Figs. 9, A-C), suggesting that the TRE binding site was required for strong AP-1 effects.

DISCUSSION

The relatively high concentration of SKF-82958 required to achieve ERα transcriptional activity in comparison to dopamine D1 receptor activation suggested that this compound was an ERα agonist, and our relative binding affinity analyses demonstrated that SKF competed with E2 for binding to either ERα or ERβ. However, the results obtained in this study demonstrate that SKF-82958 stimulates the transcriptional activity of ERα, but not ERβ, and therefore SKF-82958 is an ERα selective agonist. Intriguingly, our studies also demonstrated that SKF stimulates the transcriptional activity of AP-1, and provides evidence that in the appropriate promoter context activation of target gene expression by SKF is the combinatorial result of AP-1 and ERα activation. Understanding the role of AP-1 in SKF-dependent ERα transactivation is particularly important given the ability of SKF to activate both transcription factors. In so doing, the results of these studies provide an excellent example of how other transcription factors can seemingly enhance the potency of weak ER ligands.

Although SKF-82958 is a full agonist of dopamine D1 receptors, it failed to stimulate increases in intracellular cAMP, nor was it able to stimulate CRE-dependent gene expression in our HeLa cells. We had previously shown that dopamine treatment of HeLa cells increased cAMP levels in a dose-dependent manner *in vitro* (30), and the inability of SKF to do so here was unexpected. Although SKF-induction of cAMP in SK-N-SH cells had not been characterized, the protein kinase A (PKA) inhibitor, H89, partially blocked ERα transactivation by SKF-82958 (37), suggesting that a cAMP signaling transduction pathway was playing a role in these cells. Similarly, H89 reduced SKF induction of ER transcription activity in MCF-7 cells (27). These reports are consistent with the ability of SKF to stimulate adenylate cyclase and cAMP production via the dopamine D1 receptor (46, 47), and it is possible that in these cell models ERα transactivation by SKF is at least partially cAMP/PKA dependent and/or that H89 is inhibiting the activity of other signaling pathways able to crosstalk with ERα or AP-1. Indeed, while H89 effectively inhibits PKA, it also blocks the activity of other kinases including protein kinase B (Akt) and mitogen- and stress-activated protein kinase 1 (72).

The inability of SKF to stimulate ERβ transcriptional activity is unlikely to be due to the minor differences in the RBA of this compound for ERα and ERβ. A large number of naturally-occurring substances, as well as pharmacological and environmental agents, bind to ERs (16, 17). The crystal structures of receptors complexed with E2, diethylstilbestrol, raloxifene or 4-hydroxytamoxifen, and molecular modeling studies suggest that binding of a phenolic group to the A-ring binding pocket of the receptors' ligand binding domain is a common feature (14, 73-75). While SKF-82958 does not possess a simple phenolic ring characteristic of many ER ligands (Fig. 1A), it does have a hydroxy-phenolic ring with a large, bulky chlorine substituent. Based on the ability of C(2) substituted derivatives of E2 and estrone (2-hydroxyestradiol and 2-hydroxyestrone, respectively) to have severely reduced RBA for ERs (16, 17) and the additional chlorine atom on SKF-82958, it was unexpected that SKF would inhibit E2 occupancy of the ligand binding pocket. This result is perhaps even more surprising in view of the inability of dopamine to bind to ERα or ERβ, since dopamine also possesses a hydroxy-phenol ring.

However, it is possible that the remainder of the dopamine molecule is of insufficient size to interact with other regions of the ligand binding pocket required for high affinity binding.

There is significant interest in identifying ER subtype selective agonists and antagonists, and several investigators have made progress in identifying and characterizing such compounds. These include a *cis*-diethyl-substituted tetrahydrochrysene which has a 4-fold preferential binding affinity for ERβ and is an ERα agonist and complete ERβ antagonist (76), and a methoxychlor metabolite that inhibits estrogen-induced ERβ activity, yet stimulates the transcriptional activity of ERα (77). Potency selective agonists have also been identified such as pyrazole, which has a 120-fold greater potency for stimulating ERα activity in comparison to ERβ (76), and A-ring reduced metabolites of the 19-nor synthetic progestins, norethisterone and Gestodene, which have at least a 100-fold greater potency for ERα in comparison to ERβ transcriptional activity (78). In addition to these compounds, differences in the ability of steroidal derivatives and non-steroidal phytoestrogens to bind to ERα and ERβ have also been reported (16, 17). Moreover, the differences in the relative agonist and antagonistic activity of several of these novel compounds has been found to correlate with changes in the conformation of the receptors and their ability to bind to SRC family coactivators (79). For instance, the ERα agonist propyl pyrazole triol induces an agonistic conformational change in ERα and promotes interaction of this receptor with SRC-1, GRIP1 and ACTR, but does not promote interaction of ERβ with these coactivators. We have demonstrated that SRC family coactivators as well as the general coactivator CBP can enhance SKF-induced ERα transactivation and this is consistent with SKF inducing a conformational change able to promote ERα-coactivator interactions.

Having established that SKF-82958 is an ERα-selective agonist, we examined the mechanism(s) by which it stimulated ERα-dependent gene expression. Deletion of the amino-terminal A/B domain of ERα indicates that the AF-1 domain is not required for SKF-82958 activation of ERα-dependent gene expression, nor is a fully functional AF-2 as demonstrated by data from the ERα-3x mutant. However,

both these mutations reduce the relative ability of ERα to activate gene expression, and the AF-1 and AF-2 regions are therefore required to yield a full response to SKF stimulation as has been shown in other contexts for E2 and SKF (20, 37). Deletion of the entire LBD confirms that SKF-induced ERα transcriptional activity involves the receptor's carboxy terminus. As noted above, mutations of the core domain of AF2 reduced, but did not block, the ability of SKF-mediated signaling pathways to activate gene expression, except when combined with deletions of the receptor's A/B domain, and this supports the supposition that SKF activation of ERα transcriptional activity requires the cooperative effects of both the amino- and carboxy-terminal domains. The inability of SKF to stimulate ERβ transcriptional activity is interesting in view of the contributions of ERα's AF-1 domain to this response and differences in the structure and relative transcriptional activity of the AF-1 domains of the two ER subtypes (11-13). It should also be noted that the lack of ERβ transactivation by SKF is not due to an inability of ERβ to functionally interact with AP-1, as we have observed this mechanism in the context of cAMP signaling pathways (K.M. Coleman & C.L. Smith, unpublished data).

Stimulation of ERα transcriptional activity by SKF is accompanied by increases in receptor phosphorylation at least equivalent to those induced in parallel experiments by E2. To the best of our knowledge, this is the first report of an ERα agonist other than E2, ICI 164,384 or 4-hydroxytamoxifen to phosphorylate the ER. While the site(s) and enzyme(s) responsible for this post-translational modification remain undefined and the importance of this phosphorylation to ERα transcriptional activity is unclear, it is intriguing to note that ER ligands and other signaling pathways able to stimulate ERα activity also induce its phosphorylation (4, 21). We have tested the ability of SKF-82958 to stimulate the activity of all the known ERα serine phosphorylation site mutants, but none of these mutations specifically blocked SKF-82958-induced activation of ERα. The requirement of the carboxy terminal region lead us to question whether or not phosphorylation of the only mapped C-terminal phosphorylation site (tyrosine[537]) contributed to this response. These studies examined the activity of a mouse ERα mutant in which mouse tyrosine[541] (which corresponds to human tyrosine[537]) had been mutated to a

phenylalanine. This mutant, which does not possess any constitutive activity (49), was very robustly activated by SKF-82958 treatment of the cells, indicating that phosphorylation of this tyrosine residue is not required for ERα transactivation by SKF-82958. Although not tested, it is possible that signal transduction pathways initiated by SKF-82958 (see below) could affect receptor-dependent gene expression by phosphorylating coactivators and altering their intrinsic transcriptional activity. For instance, 8Br-cAMP treatment of COS-1 cells phosphorylates SRC-1 and stimulates its intrinsic transcriptional activity (42). Similarly, growth factor signaling pathways increase the transcriptional activity of the GRIP1 and AIB1 coactivators (80, 81) and cAMP and MAPK signaling pathways increase CBP activity (82, 83). Thus, SKF-induced, ERα-dependent gene expression may result from SKF-induced alterations in ER and its coactivators.

The ability of SKF to stimulate AP-1 activity contributes to the ability of this compound to stimulate ERα dependent gene expression on the ERE-E1b-CAT reporter gene. Activation of AP-1, however, is insufficient to stimulate CAT activity from this reporter in cells lacking ERα (see Fig. 6C). Several lines of evidence indicate that the TRE site contributes to the magnitude of target gene expression by ERα and SKF-82958. First, this synthetic dopamine receptor agonist did activate transcription from a TRE-dependent reporter in the absence of co-transfected ER. Moreover, eliminating a functional AP-1 element ~255 bp upstream from the ERE-E1b-CAT reporter sequence, either by deletion or site-directed mutagenesis, significantly reduced the ability of SKF to stimulate ER transactivation. Interactions between ERα and c-Jun are mediated via the amino-terminus of ERα (57), and eliminating both the upstream AP-1 binding site from ERE-E1b-CAT and the ERα's AF-1 domain severely compromised the ability of SKF to activate ERα-dependent gene expression, suggesting that the A/B domain contributes to this activity through its ability to interact with AP-1 and/or accessory transcription factors which link AP-1 and ERα function.

Although steroid receptors can activate the transcription of target genes containing only their response elements and minimal promoters such as TATA boxes, natural target gene promoters are significantly more complex and contain binding sites for many different transcription factors. Regulation of target gene expression is therefore a result of the coordinate regulation of the activity of all transcription factors that can bind to a target promoter, and for this reason, it is important to examine the interaction between AP-1 and ERα. The mechanisms by which SKF enhanced activation of the TRE (coll73-CAT) reporter gene are not defined, but could be mediated by increased expression of AP-1 transcription factors and/or their activation by signal transduction pathway-induced post-translational modifications [e.g. phosphorylation (71, 84)]. However, we demonstrated that the magnitude of SKF-dependent ERα transactivation paralleled the relative levels of c-Jun expression (i.e. enhanced when c-Jun was overexpressed) confirming that SKF effects dependent on the TRE site are mediated by AP-1. There seems to be a preferential role for c-Jun in this system, since its overexpression resulted in a substantial enhancement of overall transcriptional activity, while c-Fos overexpression only modestly enhanced ERα-dependent transactivation. Alternatively, it is possible that other Fos family members may better stimulate ERα activity, analogous to the situation where the ability of E2 to stimulate or repress AP-1 activity appears to correlate with the relative expression of the Fos family member Fra-1 (85).

These effects of either c-Jun or c-Fos were greatly diminished on ERE-E1b-CAT reporters lacking the upstream TRE site. This is important since it suggests that AP-1 interaction with ERα in the absence of TRE DNA binding site makes very modest contributions to ERα-dependent gene expression. These relationships were particularly well demonstrated when SKF-dependent ERα transactivation of the ERE-E1b-CAT TRE site mutants was compared in the presence of wild type ER versus the ER mutant lacking the AF-1 domain (Fig. 8). Under these conditions, which limit the contribution of AP-1 both through its DNA binding site and through protein-protein interactions with ERα, E2- and SKF-82958-induced ERα transactivation were substantially diminished. Collectively, these observations are consistent with the hypothesis that AP-1 enhances SKF-dependent ER transactivation both by AP-1/TRE interaction and by

protein/protein interaction between the ER and AP-1 proteins. Whether this latter interaction is direct or indirectly mediated through other proteins such as coactivators is presently unknown.

The interactions between ERs and AP-1 are complex, and using reporters containing only AP-1 binding sites, other investigators have demonstrated two pathways for ER activation of AP-1-dependent gene expression [reviewed in ref. (3)]. There appears to be an AF-dependent pathway that estrogen- or antiestrogen-liganded ERα utilizes, while ERβ stimulates AP-1 activity in an AF-independent manner (57, 86). The results of our study suggest that AP-1 can stimulate the activity of ERα liganded with a weak agonistic ligand such as SKF-82958 or as expected with the full agonist, E2 (57), indicating that these two classes of transcription factors have the ability to regulate each other's transcriptional activity. This also suggests that the ability of any given ER ligand to activate receptor-dependent gene expression may vary depending on the presence of DNA binding sites for other transcription factors that can functional interact with the ER and/or that the ligand may regulate. Since ERs have been reported to functionally interact with AP-1 (discussed above), as well as Sp1, NF-Y and USF (87, 88), many possible regulatory combinations would seem to be possible, leading to complex regulation of ER-dependent gene expression. Taken together, these results suggest that the ability of pharmacological and environmental compounds to exert estrogen-like effects may need to take into account the activities from other transcription factors able to functionally interact with ERα.

Acknowledgments

REFERENCES

1. Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P., and Chambon, P. (1986) *Nature* **320,** 134-139

2. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93,** 5925-5930

3. Kushner, P. J., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiau, A. K., Uht, R. M., and Webb, P. (2000) *J. Steroid Biochem. Mol. Biol.* **74,** 311-317

4. Weigel, N. L. (1996) *Biochem. J.* **319,** 657-667

5. Tsai, M.-J. and O'Malley, B. W. (1994) *Ann. Rev. Biochem.* **63,** 451-486

6. Paige, L. A., Christensen, D. J., Gron, H., Norris, J. D., Gottlin, E. B., Padilla, K. M., Chang, C.-Y., Ballas, L. M., Hamilton, P. T., McDonnell, D. P., and Fowlkes, D. M. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 3999-4004

7. Pike, A. C., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engstrom, O., Ljunggren, J., Gustafsson, J.-A., and Carlquist, M. (1999) *EMBO J.* **18,** 4608-4618

8. Tremblay, A. and Giguère, V. (2001) *J. Steroid Biochem. Mol. Biol.* **77,** 19-27

9. Cowley, S. M., Hoare, S., Mosselman, S., and Parker, M. G. (1997) *J. Biol. Chem.* **272,** 19858-19862

10. Pace, P., Taylor, J., Suntharalingam, S., Coombes, R. C., and Ali, S. (1997) *J. Biol. Chem.* **272,** 25832-25838

11. Hall, J. M. and McDonnell, D. P. (1999) *Endocrinology* **140,** 5566-5578

12. Delaunay, F., Pettersson, K., Tujague, M., and Gustafsson, J.-Å. (2000) *Mol. Pharmacol.* **58,** 584-590

13. Cowley, S. M. and Parker, M. G. (1999) *J. Steroid Biochem. Mol. Biol.* **69,** 165-175

14. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell* **95,** 927-937

15. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) *Endocr. Rev.* **20,** 321-344

16. Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J. (1997) *Endocrinology* **138,** 863-870

17. Kuiper, G. G. J. M., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J.-Å. (1998) *Endocrinology* **139,** 4252-4263

18. Tora, L., White, J. H., Brou, C., Tasset, D. M., Webster, N. J. G., Scheer, E., and Chambon, P. (1989) *Cell* **59,** 477-487

19. Lees, J. A., Fawell, S. E., and Parker, M. G. (1989) *J. Steroid Biochem.* **34,** 33-39

20. Tzukerman, M. T., Esty, A., Santiso-Mere, D., Danielian, P., Parker, M. G., Stein, R. B., Pike, J. W., and McDonnell, D. P. (1994) *Mol. Endocrinol.* **8,** 21-30

21. Weigel, N. L. and Zhang, Y. (1997) *J. Mol. Med.* **76,** 469-79

22. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) *Science* **270,** 1491-1494

23. Bunone, G., Briand, P.-A., Miksicek, R. J., and Picard, D. (1996) *EMBO J.* **15,** 2174-2183

24. Ignar-Trowbridge, D. M., Teng, C. T., Ross, K. A., Parker, M. G., Korach, K. S., and McLachlan, J. A. (1993) *Mol. Endocrinol.* **7,** 992-998

25. Tremblay, G. B., Tremblay, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Labrie, F., and Giguere, V. (1997) *Mol. Endocrinol.* **11,** 353-365

26. Tremblay, A., Tremblay, G. B., Labrie, F., and Giguère, V. (1999) *Mol. Cell* **3,** 513-519

27. Riby, J. E., Chang, G. H. F., Firestone, G. L., and Bjeldanes, L. F. (2000) *Biochem. Pharmacol.* **60,** 167-177

28. Patrone, C., Ma, Z. Q., Pollio, G., Agrati, P., Parker, M. G., and Maggi, A. (1996) *Mol. Endocrinol.* **10,** 499-507

29. Pietras, R. J., Arboleda, J., Reese, D. M., Wongvipat, N., Pegram, M. D., Ramos, L., Gorman, C. M., Parker, M. G., Sliwkowski, M. X., and Slamon, D. J. (1995) *Oncogene* **10,** 2435-2446

30. Smith, C. L., Conneely, O. M., and O'Malley, B. W. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90,** 6120-6124

31. Power, R. F., Mani, S. K., Codina, J., Conneely, O. M., and O'Malley, B. W. (1991) *Science* **254,** 1636-1639

32. Matkovits, T. and Christakos, S. (1995) *Mol. Endocrinol.* **9,** 232-242

33. Apostolakis, E. M., Garai, J., Fox, C. F., Smith, C. L., Watson, S. J., Clark, J. H., and O'Malley, B. W. (1998) *J. Neurosci.* **16,** 4823-4834

34. Mani, S. K., Allen, J. M., Clark, J. H., Blaustein, J. D., and O'Malley, B. W. (1994) *Science* **265,** 1246-1249

35. Mani, S., Allen, J. M. C., Lydon, J. P., Mulac-Jericevic, B., Blaustein, J. D., DeMayo, F. J., Conneely, O. M., and O'Malley, B. W. (1996) *Mol. Endocrinol.* **10,** 1728-1737

36. Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., and Caron, M. G. (1998) *Physiol. Rev.* **78,** 189-225

37. Gangolli, E. A., Conneely, O. M., and O'Malley, B. W. (1997) *J. Steroid Biochem. Molec. Biol.* **61,** 1-9

38. Aronica, S. M. and Katzenellenbogen, B. S. (1993) *Mol. Endocrinol.* **7,** 743-752

39. LeGoff, P., Montano, M. M., Schodin, D. J., and Katzenellenbogen, B. S. (1994) *J. Biol. Chem.* **269(6),** 4458-4466

40. Denner, L. A., Weigel, N. L., Maxwell, B. L., Schrader, W. T., and O'Malley, B. W. (1990) *Science* **250,** 1740-1743

41. Bai, W., Rowan, B. G., Allgood, V. E., O'Malley, B. W., and Weigel, N. L. (1997) *J. Biol. Chem.* **272,** 10457-10463

42. Rowan, B. G., Garrison, N., Weigel, N. L., and O'Malley, B. W. (2000) *Mol. Cell. Biol.* **20,** 8720-8730

43. Kuhar, M. J., Ritz, M. C., and Boja, J. W. (1991) *Trends Neuroscience* **14,** 299-302

44. Katz, J. L., Kopajtic, T. A., Myers, K. A., Mitkus, R. J., and Chider, M. (1999) *J. Pharmacol. Exp. Therap.* **291,** 265-279

45. Jenner, P. (1995) *Neurology* **45,** S6-S12

46. O'Boyle, K. M., Gaitanopoulos, D. E., Brenner, M., and Waddington, J. L. (1989) *Neuropharmacology* **28,** 401-405

47. Murray, A. M. and Waddington, J. L. (1989) *Eur. J. Pharmacol.* **160,** 377-384

48. Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantx, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J.-A. (1997) *J. Clin. Endocrinol. Metab.* **82,** 4258-4265

49. White, R., Sjöberg, M., Kalkhoven, E., and Parker, M. G. (1997) *EMBO J.* **16,** 1427-1435

50. Baichwal, V. R., Park, A., and Tjian, R. (1998) *Nature* **352,** 165-8

51. Sassone-Corsi, P., Lamph, W. W., Kamps, M., and Verma, I. M. (1988) *Cell* **54,** 553-60

52. Sun, P., Lou, L., and Maurer, R. A. (1996) *J. Biol. Chem.* **271,** 3066-3073

53. Lonard, D. M., Nawaz, Z., Smith, C. L., and O'Malley, B. W. (2000) *Mol. Cell* **5,** 939-948

54. Allgood, V. E., Oakley, R. H., and Cidlowski, J. A. (1993) *J. Biol. Chem.* **268,** 20870-20876

55. Nawaz, Z., Lonard, D. M., Smith, C. L., Lev-Lehman, E., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1999) *Mol. Cell. Biol.* **19,** 1182-1189

56. Kurten, R. C. and Richards, J. S. (1989) *Endocrinology* **125,** 1345-1357

57. Webb, P., Lopez, G. N., Uht, R. M., and Kushner, P. J. (1995) *Mol. Endocrinol.* **9,** 443-456

58. Pham, T. A., Elliston, J. F., Nawaz, Z., McDonnell, D. P., Tsai, M.-J., and O'Malley, B. W. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3125-3129

59. Morgenstern, J. P. and Land, H. (1990) *Nuc. Acids Res.* **18,** 1068

60. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, G., Herrlich, P., and Karin, M. (1987) *Cell* **49,** 729-739

61. Allgood, V. E., Zhang, Y., O'Malley, B. W., and Weigel, N. L. (1997) *Biochem.* **36,** 224-232

62. Cristiano, R. J., Smith, L. C., and Woo, S. L. C. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2122-2126

63. Seed, B. and Sheen, J.-Y. (1988) *Gene* **67,** 271-277

64. Bai, W., Tullos, S., and Weigel, N. L. (1994) *Mol. Endocrinol.* **8,** 1465-1473

65. Jordan, V. C. (1984) *Pharmacol. Rev.* **36,** 245-276

66. Ali, S., Metzger, D., Bornert, J. M., and Chambon, P. (1993) *EMBO J.* **12(3),** 1153-1160

67. Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. (1994) *Mol. Endocrinol.* **8,** 1208-1214

68. Gorelova, N. A. and Yang, C. R. (2000) *J. Neurophys.* **84,** 75-87

69. Asghar, M., Hussain, T., and Lokhandwala, M. F. (2001) *Eur. J. Pharmacol.* **411,** 61-66

70. Imagawa, M., Chiu, R., and Karin, M. (1987) *Cell* **51,** 251-260

71. Karin, M., Liu, Z., and Zandi, E. (1997) *Curr. Opin. Cell Biol.* **9,** 240-246

72. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) *Biochem. J.* **351,** 95-105

73. Stauffer, S. R., Coletta, C. J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2000) *J. Med. Chem.* **43,** 4934-4947

74. Anstead, G. M., Carlson, K. E., and Katzenellenbogen, J. A. (1997) *Steroids* **62,** 268-303

75. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J.-Å., and Carlquist, M. (1997) *Nature* **389,** 753-758

76. Sun, J., Meyers, M. J., Fink, B. E., Rajendran, R., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (1999) *Endocrinology* **140,** 800

77. Gaido, K. W., Leonard, L. S., Maness, S. C., Hall, J. M., McDonnell, D. P., Saville, B., and Safe, S. (1999) *Endocrinology* **140,** 5746-5753

78. Larrea, F., García-Becerra, R., Lemus, A. E., García, G. A., Pérez-Palacios, G., Jackson, K. J., Coleman, K. M., Dace, R., Smith, C. L., and Cooney, A. J. (2001) *Endocrinology* **142,** 3791-3799

79. Kraichely, D. M., Sun, J., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (2000) *Endocrinology* **141,** 3534-3545

80. Lopez, G. N., Turck, C. W., Schaufele, F., Stallcup, M. R., and Kushner, P. J. (2001) *J. Biol. Chem.* **276,** 22177-22182

81. Font de Mora, J. F. and Brown, M. (2000) *Mol. Cell. Biol.* **20,** 5041-5047

82. Ait-Si-Ali, S., Carlisi, D., Ramirez, S., Upegui-Gonzalez, L. C., Duquet, A., Robin, P., Rudkin, B., Harel-Bellan, A., and Trouche, D. (1999) *Biochem. Biophys. Res. Comm.* **262,** 157-162

83. Chrivia, J. C., Kwok, R. P. S., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) *Nature* **365,** 855-859

84. Karin, M. and Hunter, T. (1995) *Curr. Biol.* **5,** 747-757

85. Philips, A., Teyssier, C., Galtier, F., Rivier-Covas, C., Rey, J.-M., Rochefort, H., and Chalbos, D. (1998) *Mol. Endocrinol.* **12,** 973-985

86. Webb, P., Nguyen, P., Valentine, C., Lopez, G. N., Kwok, G. R., McInerney, E., Katzenellenbogen, B. S., Enmark, E., Gustafsson, J.-Å., Nilsson, S., and Kushner, P. J. (1999) *Mol. Endocrinol.* **13,** 1672-1685

87. Wang, W., Dong, L., Saville, B., and Safe, S. (1999) *Mol. Endocrinol.* **13,** 1387

88. Xing, W. and Archer, T. K. (1998) *Mol. Endocrinol.* **12,** 1321

FIGURE LEGENDS

Figure 1: SKF-82958 activates ERα-dependent gene expression. (A) Chemical structures of the compounds used to regulate ERα activity in this study. (B) Activation of ERE-E1b-Luc target gene expression by SKF-82958 is ER dependent. HeLa cells were transfected with expression vectors for ERα (pCMV$_5$-hERβ) and β-galactosidase (pCMVβ), and the ERE-E1b-Luc reporter gene and subsequently treated with ethanol (vehicle), 1 nM E2 or 10 μM SKF-82958 (SKF) in the absence or presence of 100 nM ICI 182,780. Data represent the average of three independent experiments ± SEM. (C) SKF-82958 does not stimulate CRE-dependent transcriptional activity. HeLa cells were transfected with either a CRE-containing (p-169αCG-CAT) or CRE-minus (p-100αCG-CAT) reporter gene and subsequently treated with ethanol (Vehicle), 1 nM E2, 25 μM SKF-82958 (SKF), 1 mM 8Br-cAMP and 100 μM IBMX (cAMP) or 200 μM dopamine (DA). Activation data represent the average ± SEM of three independent experiments.

Figure 2: SKF-82958 binds to ERα and ERβ. *In vivo* hormone binding assays of (A) ERα or (B) ERβ were performed to assess the relative binding affinity of E2, SKF-82958 (SKF) or dopamine (DA) with respect to competition for [^{3}H]estradiol binding to receptor. Total [^{3}H]estradiol binding in the absence of competitor (♦) is shown for cells treated with ethanol (Veh). Values represent the average of duplicate samples from a representative experiment. Similar results were obtained in n=3-4 independent experiments.

Figure 3: SKF-82958 is an ERα selective activator of transcription. (A) HeLa cells were transfected with expression vectors for ERα (pCMV$_5$-hERα) or ERβ (pCMV$_5$-hERβ) along with ERE-E1b-Luc and pCMVβ, and subsequently treated with ethanol (Veh), 1 nM E2 or 10 μM SKF-82958. Data represent the average of four independent experiments ± SEM. (B) Downregulation of ERα expression by SKF-82958. Western blot analysis of cell extracts prepared from HeLa cells transfected with an ERα expression vector

and subsequently treated with ethanol (Veh), 1 nM E2 or 25 μM SKF-82958 (SKF). Signals were detected with H222 antibody. **(C)** HeLa cells were transfected with ERE-Elb-Luc and expression vectors for ERα and β-galactosidase along with plasmids for SRC-1e, TIF2, RAC3, CBP or the corresponding parental (empty) vector, pCR3.1. Cells were subsequently treated with ethanol (Veh), 1 nM E2 or 10 μM SKF-82958. Values represent results from an experiment performed in duplicate and repeated at least three times.

Figure 4: Comparison of wild type and Flag-tagged ERα. **(A)** Western blot analysis of extracts prepared from cells transfected with pJ3-Flag-ERα or pJ3Ω (mock). Blot was probed with anti-Flag (M2) antibody. **(B)** Western blot of wild type and Flag-ERα expressed in HeLa cells. Blot was probed with anti-hERα (H222) antibody. **(C)** Dose response curves for wild type (wt; ■) and Flag-tagged (O) ERα in HeLa cells. Cells were transfected with increasing amounts of expression vectors for wild type or Flag-tagged ERα along with ERE-Elb-Luc and CMVβgal, and subsequently treated with 1 nM E2. Data are standardized to the activity of cell lysates prepared from cells transfected with 250 ng wild type ERα, and represent the mean ± SEM of three independent experiments. **(D)** HeLa cells were transfected with 250 ng of the expression vector for each of the indicated receptor forms along with ERE-Elb-Luc and CMVβgal. Cells were treated with ethanol (Veh), 1 nM E2 or 10 μM SKF-82958 (SKF). Results are standardized to E2 values and represented the average ± SEM of three independent experiments.

Figure 5: SKF-82958 induces ERα phosphorylation. **(A)** HeLa cells transfected with expression vector for Flag-ERα (lanes 1-3) or empty vector (pJ3Ω; lane 4) were radiolabelled with [^{32}P]orthophosphate and treated with ethanol (Veh), 1 nM E2 or 25 μM SKF-82958 (SKF). Receptors were immunoprecipitated with H222 antibody, resolved by SDS-PAGE, transferred to nitrocellulose and exposed for autoradiography (*top*) and subsequently subjected to Western blot analysis with an anti-Flag

(M2) antibody (*bottom*). **(B)** Values represent the average ± SEM of relative ERα phosphorylation determined in 4 independent experiments.

Figure 6: The AF1 and AF2 domains of ERα are required for optimal activation of transcription by SKF-82958. **(A)** HeLa cells were transfected with expression vectors for wild type (pCMV$_5$-hERα) or various alanine mutants of human ERα or a tyrosine to phenylalanine mutant of mouse ERα (Y541F) along with ERE-E1b-Luc and CMVβgal. Data are presented as the average ± SEM of 3-5 independent experiments. **(B)** Schematic of ER mutants used in experiments shown in panel C. The location of the D538A/E542A/D545A amino acid mutations are indicated by '•'. **(C)** HeLa cells were transfected with pRST7 (empty plasmid) or pRST7 expression vectors for wild type ERα (wt), ERα-3x (3x), ERα-N282G (N282G), ERα-179C (179C) or ERα-179C-3x (179C-3x) along with ERE-E1b-Luc and pCMVβ. Data are presented as the average ± SEM of 3 experiments. For panels A & C, cells were treated with ethanol (Veh), 1 nM E2 or 10 μM SKF-82958 (SKF). The activity of wild type ERα in the presence of 1 nM E2 was defined as 100.

Figure 7: SKF-82958 modestly activates gene expression from a TPA-responsive element (TRE). **(A)** Schematic representation of the coll73-CAT and coll60-CAT reporter genes used in these experiments. **(B)** HeLa cells were plated at a low density (2 x 10^5 cells/well), switched to media containing 0.5% sFBS and transfected with coll73-CAT or coll60-CAT reporter plasmid. Cells were treated with ethanol (vehicle), 1 nM E2, 100 nM TPA, 10 μM SKF-82958 or 100 nM 4HT. Values represent mean ± SEM for n=4-5 experiments and are expressed as fold induction relative to vehicle-treated cells transfected with coll73-CAT.

Figure 8. An upstream TPA responsive element (TRE) enhances SKF-82958 activation of ERα-dependent gene expression. **(A)** Schematic representation of reporter genes used in these experiments. HeLa cells were transfected with expression vectors for **(B)** wild type ER (pSVMT-wtER) or **(C)** ERα-

179C (pRST7-hERα-179C) along with ERE-E1b-CAT reporter genes encoding a putative AP-1 responsive element (TRE-ERE), or lacking this site through deletion (□*Nde-Eco*-ERE) or mutation (mTRE-ERE). Cells were treated with the ethanol (Veh), 1 nM E2 or 10 µM SKF-82958. Bars represent mean ± SEM for n=4-6 independent experiments and values are expressed relative to the CAT activity induced by E_2 treatment from the intact TRE-ERE-E1b-CAT reporter in each experiment.

Figure 9. Overexpression of c-Jun enhances ER activity stimulated by E2 or SKF-82958. HeLa cells were co-transfected with increasing concentrations of expression plasmid for **(A)** c-Jun **(B)** c-Fos or **(C)** equivalent amounts of c-Jun and c-Fos along with pSVMT-wtER and ERE-E1b-CAT reporter genes with (TRE-ERE) or without (□*Nde1-Eco*-ERE) a TRE. Total DNA levels were normalized in each group by co-transfecting appropriate levels of the empty plasmid pRSV-Not. Transfections were done 6h prior to addition of the indicated agonists, with harvest following 18h thereafter. Cells were treated with ethanol (vehicle), 1 nM E2 or 10 µM SKF-82958 (SKF). Bars represent mean ± SEM for n=3 independent experiments and values are expressed relative to the CAT activity (100) induced by E2 treatment from ERE-E1b-CAT in each experiment. ANOVA indicated that (a) c-Jun overexpression, both in the presence and absence of co-transfected c-Fos, significantly elevated basal ($p<0.001$) and E_2- ($p<0.01$) and SKF-82958-induced ($p<0.001$) transcriptional activation from ERE-E1b-CAT, but not from the TRE deletion mutant and (b) c-Fos overexpression resulted in modestly significant ($p<0.05$) increases in E_2- and SKF-82958-induced transcriptional activity from the intact reporter.

A

Dopamine

SKF-82958

Estradiol

B

Relative ER Activity

- □ - ICI
- ■ +ICI

Vehicle   E2   SKF

C

Relative CAT Activity

- □ p-169αCG-CAT
- ■ p-100αCG-CAT

Vehicle   E2   SKF   cAMP   DA
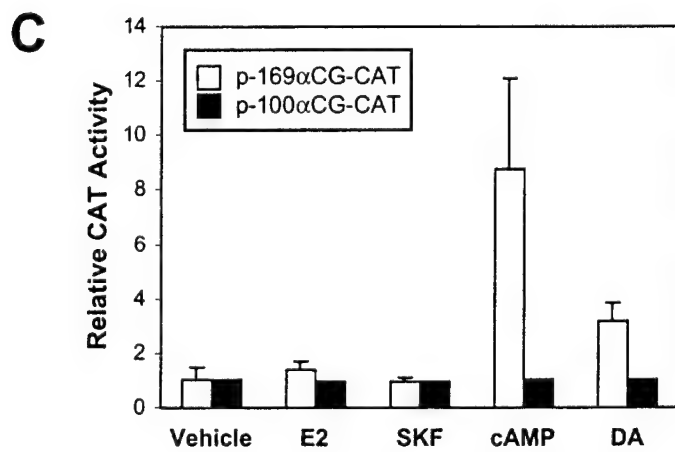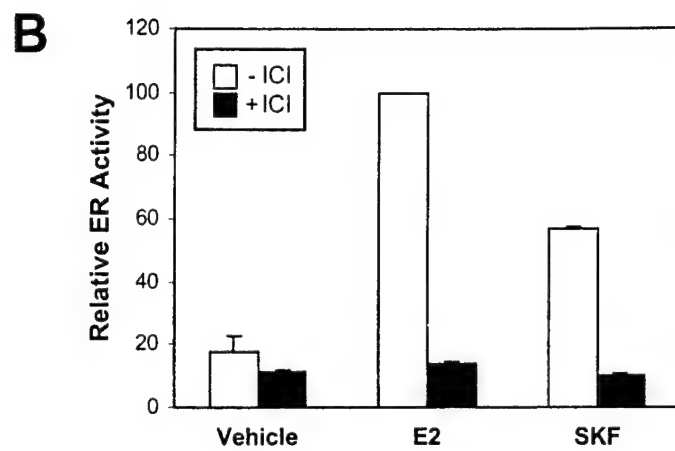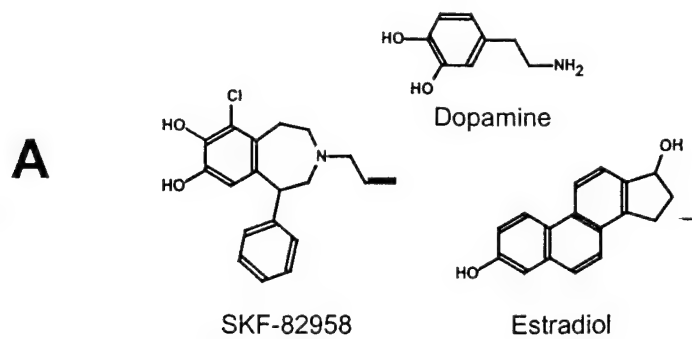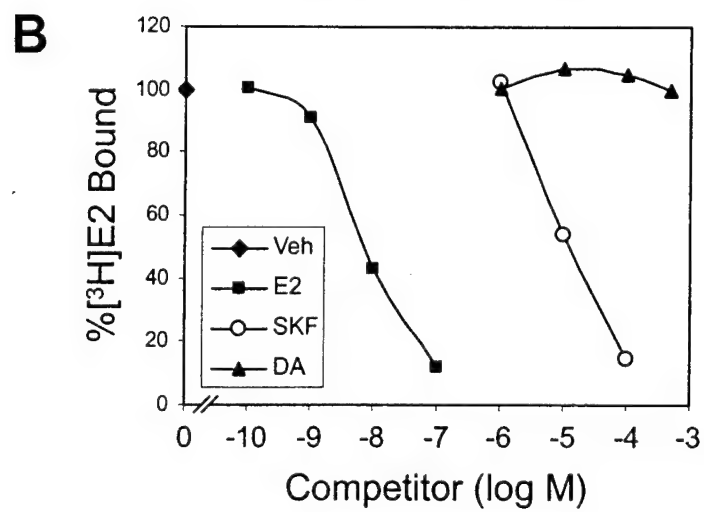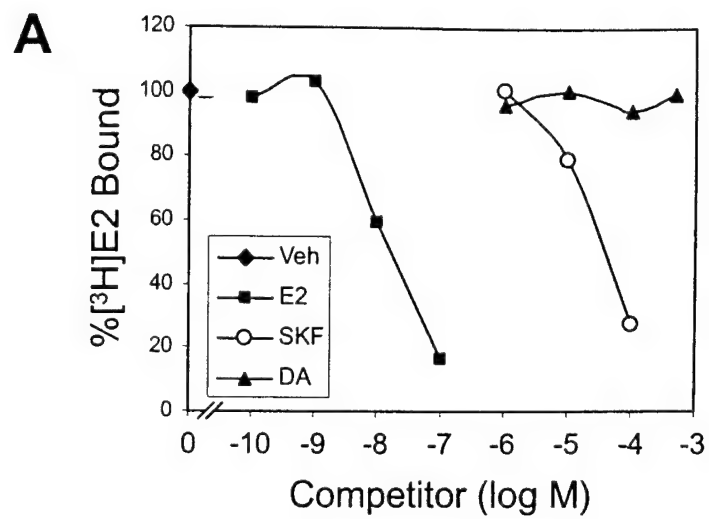
Figure 1

Figure 2

Figure 3

Figure 4

A

	Veh	E2	SKF	mock
^{32}P				
M2 Ab				
	1	2	3	4

B

Rel. ER Phosphorylation

Veh   E2   SKF

Figure 5

Figure 6

A.

Coll73-CAT [TRE]——[CAT]

Coll60-CAT ——[CAT]
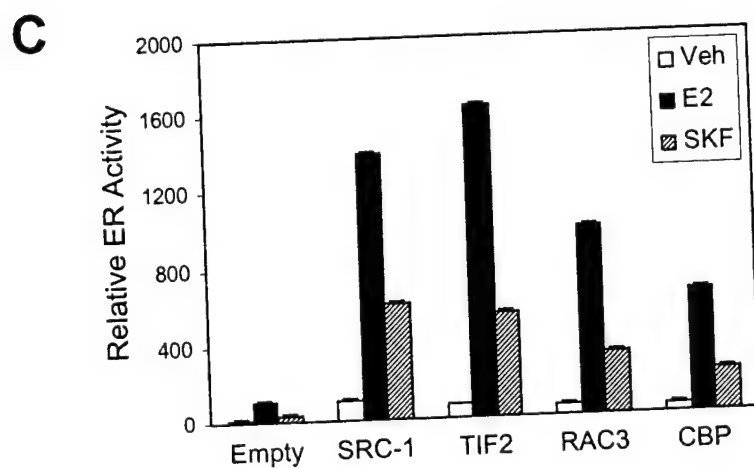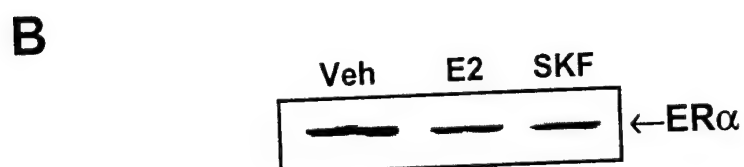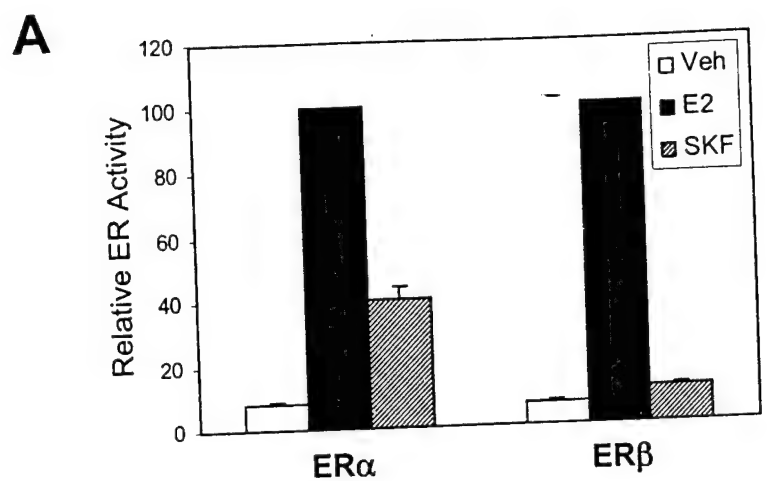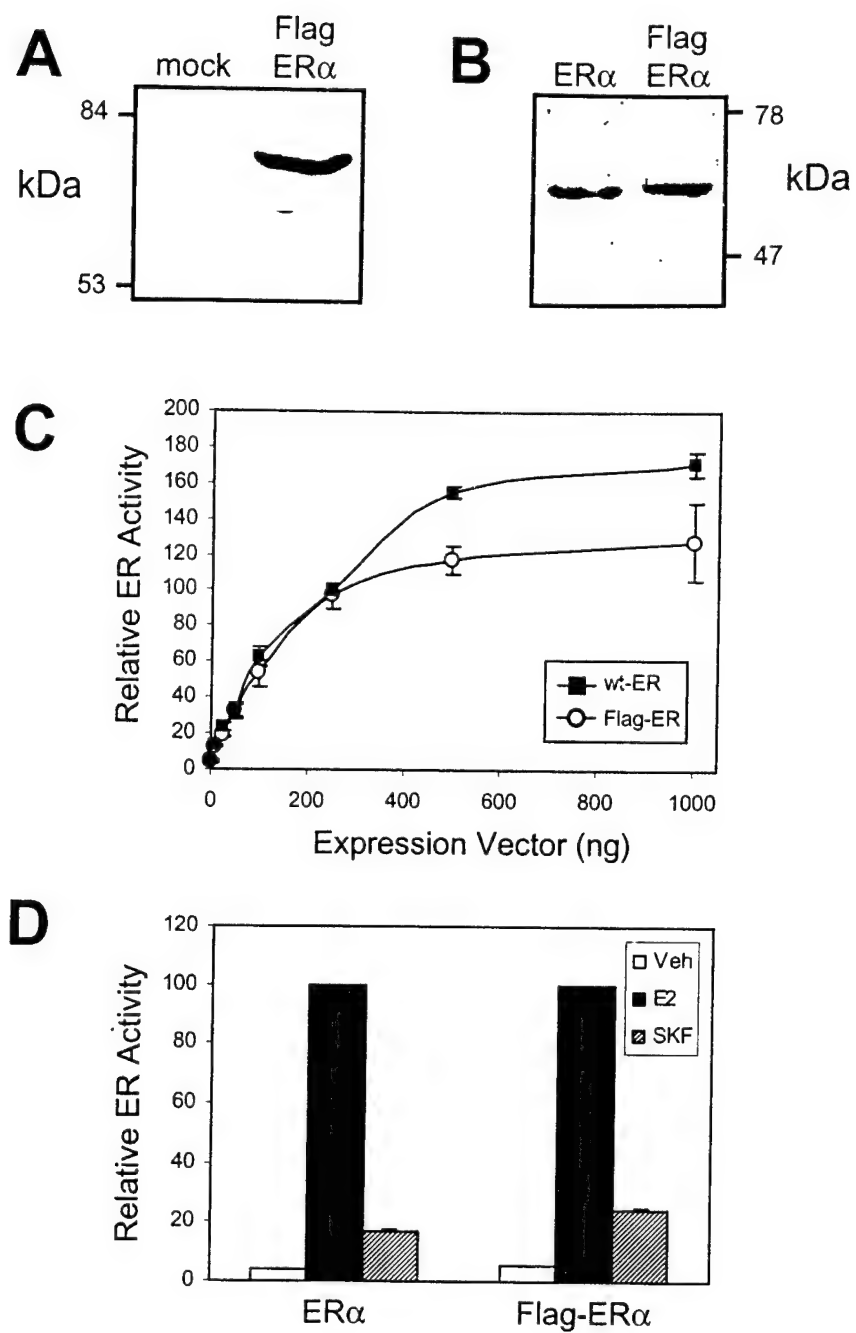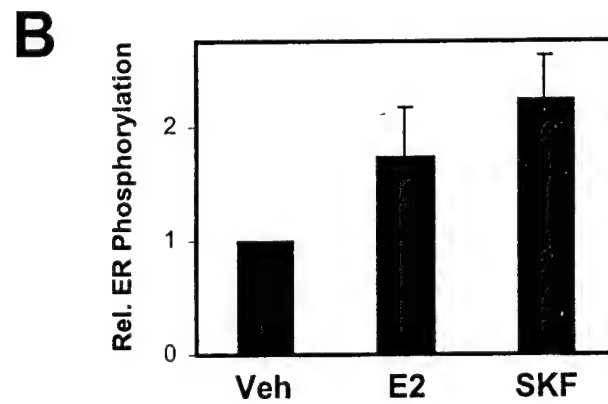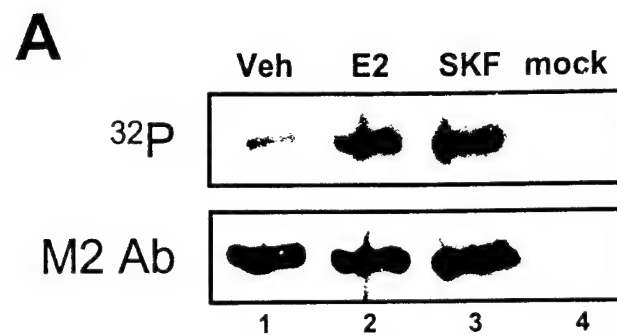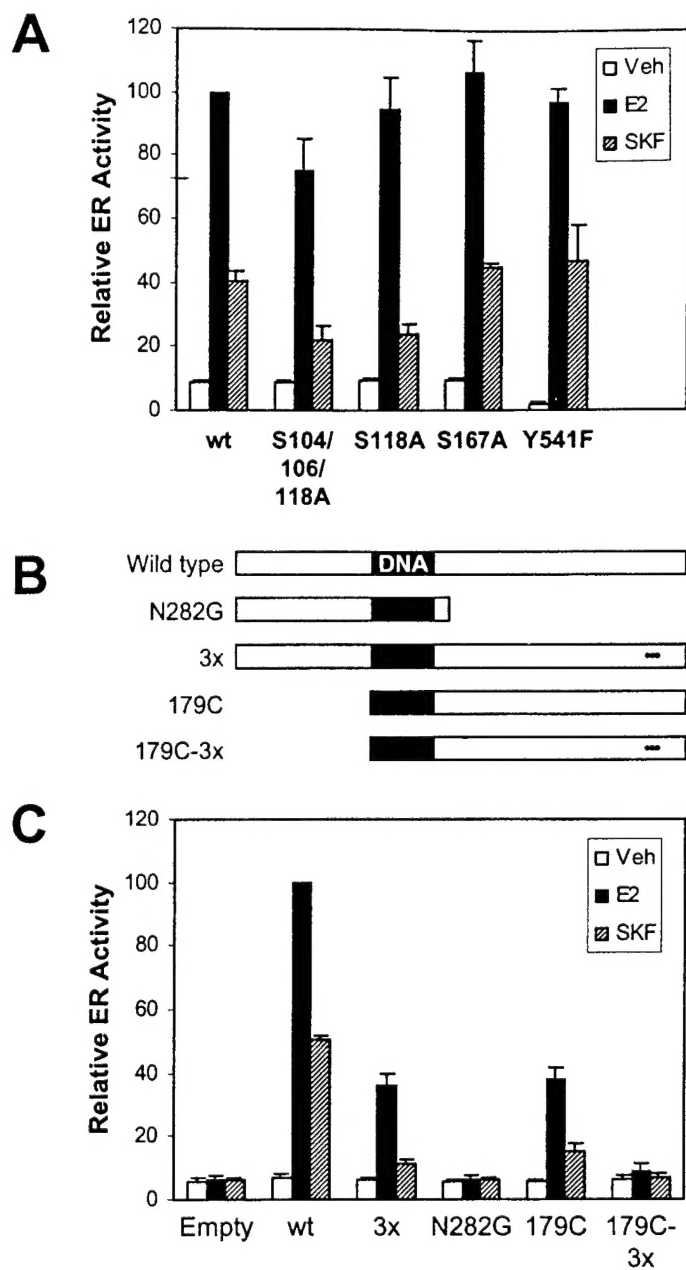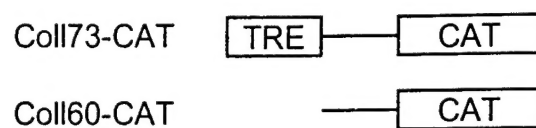
B.
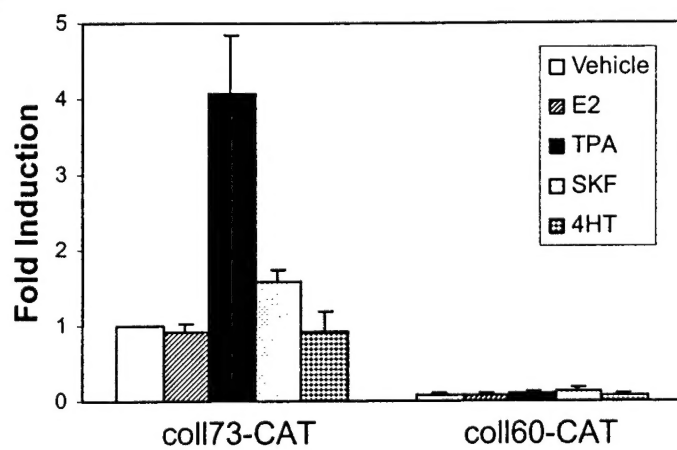
Figure 7

Figure 8

Figure 9